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Engineering durable late blight resistance to protect solanaceous plants

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Engineering durable late blight resistance to protect solanaceous plants

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DECLARATION

The results presented here are of investigations conducted by myself. Work other than my own is clearly identified with references to relevant researchers and/or their publications. I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or any other university.

Laura J. Stevens

We certify that Laura Stevens has fulfilled the relevant Ordinance and Regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

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PUBLICATIONS ARISING FROM THIS PROJECT

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ABSTRACT

Phytophthora infestans, the oomycete pathogen responsible for late blight of potato and tomato, is regarded as the biggest threat to global potato production and is thought to cost the industry around £6 billion annually. Traditionally, fungicides have been used to control the disease, but this is both economically and environmentally costly, as multiple chemical applications may be required during a single growing season. *P. infestans* has rapidly overcome genetic resistances introduced into cultivated potato from wild species. This provides the rationale for developing artificial resistance genes to create durable resistance to late blight disease.

Phytophthora species secrete essential effectors into plant cells that target critical host cellular mechanisms to promote disease. One such *P. infestans* effector is AVR3a^{KI} which is recognised by the potato R3a protein, a member of the CC-NB-LRR type resistance gene family. However, the closely related virulent form, AVR3a^{EM}, which is homozygous in more than 70% of wild *P. infestans* isolates, evades this recognition.

Domain swapping experiments have revealed that the LRR domain of R3a is involved in recognition of AVR3a^{KI}, as the CC-NB domain of an R3a-paralog which does not mediate recognition of AVR3a^{KI}, is able to induce a HR when combined with the LRR of wild-type R3a. However, a chimeric protein consisting of the CC-NB domain of a more distantly-related homolog of R3a and the LRR of domain of R3a, is unable to recognise AVR3a^{KI}, suggesting that function is achieved only when the different domains of an R protein are attuned to recognition and signalling.

Gain-of-function variants of *R3a* (*R3a**), engineered by an iterative process of error-prone PCR, DNA fragmentation, re-assembly of the leucine rich repeat (LRR)-encoding

region of *R3a*, are able to recognise both forms of AVR3a. This gain-of-recognition is accompanied by a gain-of-mechanism, as shown by a cellular re-localisation from the cytoplasm to prevacuolar compartments upon perception of recognised effector forms. However, R3a* variants do not confer resistance to AVR3a^{EM}-carrying isolates of *P. infestans*.

Future efforts will target the NB-ARC domain of R3a, in a bid to fine-tune the intra-cellular signalling of gain-of-recognition R3a* variants. It is hoped that a shuffled *R3a** gene, capable of conferring resistance to *P. infestans* isolates harbouring AVR3a^{EM}, will provide durable late blight resistance when deployed in the field in combination with other mechanistically different R proteins.

ABBREVIATIONS

µg	Microgram
µl	Microlitre
µm	Micrometre/micron
Avr	Avirulence
BiFC	Bi-molecular fluorescence complementation
bp	Base pairs
BF	Bright field
CATHB	Cathepsin B
CBEL	Cellulose binding elicitor lectin
CFP	Cyan fluorescence protein
CRN	Crinkling and necrosis (Crinkler effector)
DAMP	Danger-associated molecular pattern
DBD	DNA binding domain
dpi	Days post-infiltration/inoculation
EPIC, EPI	Extracellular protease inhibitor
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
flg22	Flagellin
FLS2, EFR	Plant receptor kinases
GM	Genetically modified
h	Hours
HR	Hypersensitive response
HSP90	Heat shock protein 90
ICD	INF1 cell death
INF1	Elicitin infestans 1
Kb	Kilobase
kDA	Kilodaltons
LRR	Leucine rich repeat
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
ml	Millilitre
mm	Millimetre
n	Number
NBS	Nucleotide-binding site
NHR	Non-host resistance
NLP	Nep1-like protein
nm	Nanometers
OD	Optical density
p	Statistical value
p19	Silencing suppressor p19
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI3P	Phosphatidylinositol-3-phosphate
PIP	Phosphatidylinositol monophosphate
PRR	Pattern recognition receptors
PTI	PAMP-triggered immunity

R	Resistance
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic acids
ROS	Reactive oxygen species
rpm	Revolutions per minute
S	Susceptibility
SCR	Small cysteine rich
SD	Standard deviation
$SE_{(M)}$	Standard error (of the mean)
SGT1	Suppressor of the G2 allele of skp1
SNP	Single nucleotide polymorphism
T3SS	Type-three secretion system
TLR	Toll-like receptors
TM	Transmembrane
TRV	Tobacco rattle virus
UV	Ultra violet
VIGS	Virus-induced gene silencing
WL	White light
YFP	Yellow fluorescence protein
Y2H	Yeast-2-hybrid

CHAPTER 1

INTRODUCTION

1.1 Molecular plant-pathogen interactions

Although plants are unable to move to escape pathogen attack, they are not unable to defend themselves. The first obstacle faced by any pathogen wishing to colonise a potential host plant are non-specialised, pre-formed physical barriers such as the waxy cuticle and the plant cell wall (Dangl and Jones, 2001). In addition to physical obstructions, invading bacteria, viruses, fungi, oomycetes, nematodes and insects all have to contend with and detoxify constitutively active antimicrobials (phytoanticipins) produced by plants in readiness for pathogen attack (VanEtten *et al*., 1994). If a pathogen is successful in breaking through a plant's pre-formed barriers, it will fall under the spotlight of the plant non-self surveillance system, which inevitably leads to inducible immune responses (Nürnberger and Lipka, 2005; Ingle *et al*., 2006). The plant immune system has been the focus of an intense research effort and has proven to be a multi-layered arrangement of inducible defences against pathogen invasion (**Figure 1.1**) (Jones and Dangl, 2006; Hein *et al*., 2009).

1.1.1 Pattern-Triggered Immunity (PTI)

The first inducible defence mechanism of plants is activated following the perception of microbe- or pathogen-associated molecular patterns (MAMPS or PAMPS) which are conserved molecules usually secreted or displayed on the surface of all microbes.

These invoke pattern-triggered immunity (PTI) upon detection by host pattern recognition receptors (PRRs) (Jones and Dangle, 2006). Bacterial PAMPs include flagellin, elongation factor Tu (EF-Tu), and cold shock proteins (Jones and Dangl 2006), whilst chitin is recognised as a fungal PAMP, along with β-glucans which are also found in oomycetes (Ingle *et al*., 2006). Other oomycete PAMPS include the elicitin INF1 and cellulose binding elicitor lectin (CBEL) (Kamoun, 2006; Hein *et al*., 2009). Plants also produce endogenous elicitors known as damage-associated molecular patterns (DAMPS). DAMPS are often the degradation products of lytic enzymes, released by pathogens in order to break down physical barriers in the plant host. Some examples include cell wall fragments, cutin monomers and peptides, which are released into the apoplast and are able to induce immune responses in the plant in a manner similar to PAMPS (Boller and Felix, 2009).

A particular PAMP is usually an essential molecule to the microbes that expresses it and is absent from the potential host (Ingle *et al*., 2006). As these molecules are essential and well-conserved, they cannot be readily dispensed with and are therefore targets for recognition by the host (Jones and Dangl, 2006). Host PRRs have proven difficult to identify (Zipfel, 2008), but a number have been characterised in recent years, with several receptor-like kinases (RLKs) and receptor-like proteins (RLPs) identified as having a role in PTI (Shiu and Bleecker, 2001; 2003). RLKs are surface-localised, membrane-spanning proteins that signal via a cytoplasmic kinase domain and have diverse extracellular domains for ligand-binding (Greef *et al*., 2012).

The best-characterised RLK PRR is FLS2 (flagellin sensitive 2) which detects the bacterial flg22 epitope (Gómez-Gómez and Boller, 2000; Chinchilla *et al*., 2006). *FLS2* encodes a receptor-like kinase and is made up of a signal peptide, an extracellular

ligand binding domain, a single membrane-spanning region and an intracellular serine/threonine kinase domain (Shiu and Bleecker, 2001). It is thought that plant PRRs function in a manner similar to the Toll- and Toll-like receptors (TLRs) of the adaptive immune systems of mammals (Ingle *et al*., 2006). Perception of the PAMP lipopolysaccharide (LPS), the major constituent of the outer cell envelope of Gram-negative bacteria, involves a cascade of receptors in mammals (Tan and Kagan, 2014). Recently, Ranf *et al*. (2015) identified the plasma-membrane receptor kinase LORE (lipooligosaccharide-specific reduced elicitation) that is required for responsiveness to LPS in plants (Zipfel *et al*., 2015). LORE is a lysin motif-containing PRR, which requires the action of the hydrolase LYS1 enzyme, whose activity releases soluble, immunogenic peptidoglycan fragments for detection by LORE (Ranf *et al*., 2015; Zipfel, 2015).

The receptor-like protein ELR (elicitin response) from the wild potato *Solanum microdontum* associates with BAK1/SERK3 to recognise the elicitin domain of conserved, extracellular *Phytophthora* elicitor proteins (Du *et al*., 2015). The same authors were able to demonstrate that transfer of ELR to *S. tuberosum* increased resistance to *P. infestans* (Du *et al*., 2015). In a separate study, the *Brassicaceae*-specific PRR EFR from *Arabidopsis thaliana*, conferring responsiveness to bacterial elongation factor Tu (EF-Tu), was transferred to the two solanaceous species *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) (Lacombe *et al*., 2010). Transgenic EFR plants produced ROS upon treatment with elf18, the eliciting isotope of EF-Tu, whilst the expression of defence-marker genes were induced in these plants (Lacombe *et al*., 2010). These studies highlight the possibility that broad-spectrum disease resistance could be engineered by the transfer and stacking of PRRs.

The PTI responses caused by the PRR-mediated perception of PAMPs include nitric oxide (NO) production, mitogen-activated protein kinase (MAPK) cascades which involve the sequential transfer of phosphate groups to downstream targets, the production of reactive oxygen species (ROS), deposition of callose in the cell wall and WRKY transcription factor-mediated induction of defence genes (Ingle *et al*., 2006).

The fact that the majority of plants are resistant to most plant pathogenic microbes forms the central tenet of the theory of non-host resistance (NHR) (Schulze-Lefert and Panstruga, 2011). NHR can be defined as the resistance exhibited by a plant species to all genetic variants of a non-adapted pathogen species (Stam *et al*., 2014). The NHR model suggests that in plant species that are evolutionarily distantly related to one another, resistance to a common pathogen is predominantly triggered by suites of PRRs leading to PTI (Schulze-Lefert and Panstruga, 2011). However, it is thought that the relative contribution of resistance proteins to NHR increases the more closely related plant species are to one another (Schulze-Lefert and Panstruga, 2011). The concept of effectors and resistance proteins are discussed in the following sections.

1.1.2 Effector-Triggered Susceptibility (ETS)

Adapted pathogens are able to suppress PTI by secreting protein effectors into host plant cells in a process known as Effector-Triggered Susceptibility (ETS). Effectors function by manipulating host processes and perturbing downstream signalling of plant defence responses in order to promote a cellular environment conducive for pathogen virulence (Fawke *et al*., 2015).

One well-studied class of effector molecules are the oomycete RXLR effectors, so called because of the arginine-any amino acid-leucine-arginine motif found in their amino acid sequences, often followed by an EER motif, both of which are essential for translocation into the host (Rehmany *et al.,* 2005; Whisson *et al*., 2007). RXLR effectors from *Phytophthora infestans* will be discussed in greater detail in **Section 1.3.3**. Other well-known groups of effectors are the bacterial type III effectors, which are delivered into host cells via the Type III Secretion System (T3SS) (Grant *et al*., 2006) and the Crinkling and Necrosis (CRN) effectors from *Phytophthora* species (Stam *et al*., 2013).

1.1.3 Effector-Triggered Immunity (ETI)

Resistance (R) proteins of host plants form a secondary line of inducible defences, as these proteins directly or indirectly detect pathogen effectors (then termed avirulence [AVR] proteins) and mediate effector-triggered immunity (ETI). The direct and indirect detection of effector proteins will be discussed in **Section 1.2.3**. One effect often associated with ETI is a type of programmed cell death (PCD) known as the hypersensitive response (HR), which prevents further spread of disease in plant tissues (Vleeshouwers *et al*., 2000). ETI triggers many of the same effects involved in PTI, such as ROS production and the activation of mitogen-activated protein kinase (MAPK) cascades, but ETI is typically a faster and stronger response (Coll *et al*., 2011). The HR is tightly genetically regulated and is associated with cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption (Coll *et al*., 2011).

The vast majority of resistance proteins are from the nucleotide-binding (NB) leucine-rich repeat (LRR) class of immune receptors (van der Biezen and Jones, 1998a). Structurally, NB-LRRs are modular proteins made up of well-defined domains and can be subdivided into two groups dependent on the domain present at the N-terminus. TIR-NB-LRRs (TNLs) have N-terminal domains with homology to the *Drosophila* toll and human interleukin-1 receptor (TIR), whilst some CC-NB-LRRs (CNLs), but not all, have a predicted coiled-coil (CC) domain within the protein's N-terminus (McHale *et al*., 2006). Some NB-LRRs which fall into the CNL class of receptors are often referred to as non-TIR-type NB-LRRs (nTNLs) as it is difficult to identify specific structural domains in the N-termini of these proteins (Jacob *et al*., 2013). NB-LRR proteins will be discussed in more detail in **Section 1.2**.

Some examples of resistance proteins that are not classified as either TIR- or CC-NB-LRR proteins are the tomato Cf proteins which confer resistance to the fungal pathogen *Cladosporium fulvum* (Hammond-Kosack *et al*., 1994). Cf proteins can be classed alongside PRRs as they are receptor-like proteins (RLPs) and have an extracellular LRR domain, a single transmembrane (TM) domain, and a small cytoplasmic tail (Kruijt *et al*., 2005). The tomato resistance protein PTO has a serine/threonine kinase catalytic domain and a myristoylation motif (Martin *et al*., 1993), but requires a binding partner, PRF, to mediate resistance to the strain of *Pseudomonas syringae* pv. tomato expressing the *avrPto* gene (Salmeron *et al*., 1996).

1.1.4 Effector-Triggered Susceptibility 2 (ETS2)

Avoiding recognition by a host is essential for a successful pathogen, so it is therefore predictable that the loss of effectors from pathogen repertoires occurs. The *P. infestans* effector AVR4 is recognised by the potato resistance protein R4, leading to a HR (van Poppel *et al*., 2008). However, *P. infestans* isolates that are virulent on *R4*-carrying plants have been shown to possess non-functional or truncated alleles of *avr4* (van Poppel *et al*., 2008). Effector-Triggered Susceptibility 2 (ETS2) is a result of co-evolution between pathogens and their hosts, with pathogens shedding effectors or evolving additional effectors that suppress host recognition or downstream signalling events (Hein *et al*., 2009).

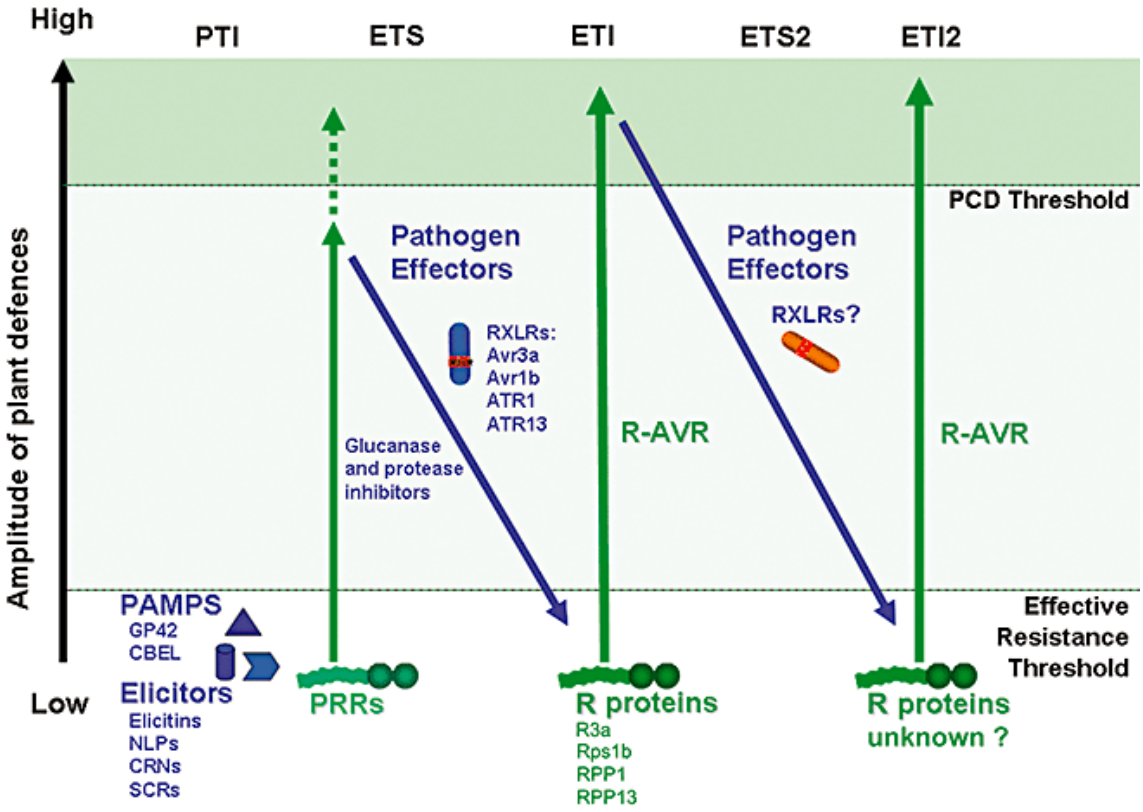

Figure 1.1: The zig-zag-zig in oomycete-plant interactions (reproduced from Hein *et al*., 2009). The amplitude of plant defence is shown on the y axis and the threshold for activation of host PCD is also indicated. Characterized oomycete pathogen-associated molecular patterns (PAMPs) and other elicitors of PAMP-triggered immunity (PTI) and necrosis are shown [represented by a dotted arrow extending PTI beyond the threshold for host programmed cell death (PCD)]. Oomycetes secrete effectors into the host, which contribute to effector triggered susceptibility (ETS), whilst host resistance proteins directly or indirectly detect oomycete effectors, leading to effector-triggered immunity (ETI). Further levels of oomycete-host interactions (ETS2 and ETI2) are indicated. (CBEL - cellulose-binding elicitor lectin; CRN - crinkling and necrosis; NLP - Nep1-like protein; PRR - pattern recognition receptor; R - resistance; SCR - small cysteine-rich)

1.2 Resistance (*R*) genes and proteins

The idea of the gene-for-gene hypothesis was first proposed by Harold Flor, based on his observations that particular lines of flax were resistant to races of *Melampsora lini* (flax rust) that had inherited certain avirulence genes (Flor, 1971). The theory revolves around the central premise that a pathogen-derived gene product, which is now regarded as an (avirulence) effector molecule, is sensed by a corresponding protein within a plant host. Effectors have been shown to be detected directly and indirectly by plant sensor proteins and the gene-for-gene hypothesis has been extended to include genes-for gene, gene for genes and genes-for-genes detection mechanisms (Gassmann and Bhattacharjee, 2012). Indeed, indirect detection of effectors has previously been described as the "guard model", which hypothesises that host R proteins monitor (guard) host effector targets. Modifications of these targets result in the activation of the R protein, triggering disease resistance signalling (van der Biezen and Jones, 1998; Dangl and Jones, 2001). (See **Section 1.2.3**).

1.2.1 *R* gene evolution

Plants are under immense pressure to maintain resistance to microbial pathogens and most do this by having a large complement of *R* genes. Diversity of NB-LRR genes is thought to be created by genome duplications, gene conversions, unequal crossing over and ectopic recombination (Marone *et al*., 2013). On the other side of this co-evolutionary relationship are the pathogens which are under evolutionary pressure to evade recognition by plant *R* genes, whilst remaining virulent to the host. The vast majority of plant microbial pathogens have very rapid life cycles, being able to produce multiple generations in a single plant growing season.

R genes can be broadly classed as either fast- or slow-evolving, referred to as Type I or Type II *R* genes, respectively. Type I *R* genes are regarded as fast-evolving, with frequent sequence exchanges between paralogs, giving rise to increased diversity between haplotypes and high homology between paralogs (Friedman and Baker, 2007). The more slowly evolving 'type II' genes are characterised by fewer sequence exchanges between paralogs, resulting in higher levels of orthology and synteny (Friedman and Baker, 2007). Michelmore and Meyers (1998) proposed that plant *R* genes evolve mainly through divergent evolution of individual genes in a birth-and-death process, with inter-allelic recombination, single point mutations, pseudogenes and transposable elements all driving this evolution.

1.2.2 Structure and function of NB-LRR resistance proteins

The best described family of *R* genes is the nucleotide-binding (NB) leucine-rich repeat (LRR) class of immune receptors (van der Biezen and Jones, 1998a). Within the NB domain, often referred to as the NB-ARC domain, sits a large conserved complex named after the first three proteins it was identified in; human apoptotic protease-activating factor-1 (APAF-1), plant R proteins and *Caenorhabditis elegans* death-4 protein (CED-4) (van der Biezen and Jones, 1998b). Several other domains are found within the NB-ARC domain, including the kinase 1a, (or P-loop domain), 2 and 3a domains, as well as other short conserved motifs of unknown function (van der Biezen and Jones, 1998b).

Models for NB-LRR R proteins suggest that the NBS domain is involved in downstream signalling leading to immune responses, whilst the LRR domain is associated with

recognition specificity of effector proteins (Jones and Takemoto *et al*., 2004). There is evidence that the LRR domain is also involved in repressing inappropriate activation of the NBS domain to prevent run-away cell death (Belkhadir *et al*., 2004).

As yet, there are no three dimensional crystal structures determined for a full-length plant resistance protein. However, predicted 3D models for the TIR domain of NBS-LRR resistance protein L6 of flax (*Linum usitatissimum*) and the NB-ARC domain of I-2 from tomato have been proposed (Bernoux *et al*., 2011; van Ooijen *et al.,* 2008). The NB-ARC domain is thought to act as a 'molecular switch', with the NB subdomain behaving as a catalyst and the ARC1 domain required as a protein scaffold for interaction with the LRR. The ARC2 subdomain acts as a regulator which transduces LRR-mediated effector recognition into downstream immune responses (van Ooijen *et al*., 2008). These models are in agreement with findings from a study of the crystal structures of APAF-1 and CED-4 (Takken *et al*., 2006). The crystal structure of the L6 TIR domain has revealed that there are distinct regions within this domain involved in self-association, signalling, and autoregulation (Bernoux *et al*., 2011).

Extensive mutational analyses of some R proteins has highlighted that the LRR domain has a high degree of tolerance for substitutions, which may drive the evolution of recognition specificity (Dinesh-Kumar *et al*., 2000, Axtell *et al*., 2001; Tornero *et al*., 2002). Variability within the LRR domain of NB-LRR proteins has proved difficult for 3D structure modelling and prediction. Much of what is known about the structure of LRR domains of NB-LRRs comes from the structure of leucine-rich repeats within a porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1993) and subsequent homology modelling of different LRR structures held in protein databases. LRRs are thought to be compact, horseshoe-shaped structures when inactive (Takken and Goverse, 2012).

Diversity studies of RPS2 suggest that the LRR sequence can also determine interactions with other host factors and so may not only play a role in recognition (Banerjee *et al*., 2001).

The 'molecular switch' model predicts that recognition of an effector by an LRR domain induces a conformational change within the NB-ARC domain, allowing ADP to be exchanged for ATP. The binding of ATP activates the R protein, triggering a conformational change in the NB effector domain, thus allowing the R protein to mount an immune response (Takken *et al*., 2006).

1.2.3 Mechanisms behind R protein-mediated effector recognition

There are two modes of recognition of AVR proteins by R proteins; direct and indirect. Direct recognition of the flax rust AVRL567 protein by the flax L5, L6, and L7 R proteins inside the plant cell has driven the diversifying selection of 12 allelic polymorphic variants of AVR567 (Dodds *et al*., 2006). However, direct recognition by R proteins is thought to be rare and it is considered more likely that effectors are indirectly recognised by plant host R proteins (McDowell and Simon, 2006). Indirect recognition of AVR genes can be described by one of two models, namely the guard and decoy hypotheses.

The guard model was first described by van der Biezen and Jones (1998a) to explain recognition of *Pseudomonas syringae* pv. *tomato* effector AVRPto by Pto and Prf of tomato. In this model, a plant R protein (the guard) monitors the status of another host protein (the guardee), which is presumed to be indispensable for the virulence function of a pathogen. In the absence of the host R protein, an interaction between

the effector and the host target proteins would contribute to pathogen fitness (Jones and Takemoto, 2004; van der Hoorn and Kamoun, 2008). The R protein is able to recognise modifications to the guardee protein caused by an effector molecule. Alternatively, the R protein could be displaced from, or recruited to the guardee/effector protein complex, in order to trigger defence.

The guard model allows the recognition of a number of effectors by just a single R protein and could explain the perceived dearth of *R* genes in plant genomes. Fewer R proteins would be required to detect changes to a number of key regulators of critical pathways. For example, the RIN4 protein from *Arabidopsis* is targeted by three unrelated effectors, AvrB, AvrRPM1 and AvrRpt2 from *Pseudomonas syringae.* AvrB and AvrRpm1 mediate phosphorylation of RIN4 to achieve immune suppression (Chung *et al*., 2001). However, this modification of RIN4 is detected and responded to by the R protein RPM1 (Mackey *et al*., 2002; 2003), triggering a HR. A third effector, AvrRpt2, cleaves RIN4 into separate domains, which are thought to function to suppress PTI (Afzal *et al*., 2011). Cleavage of RIN4 inhibits recognition by RPM1, but is detected by the R protein RPS2 (Mackay *et al*., 2003).

Pathogen virulence targets, or guardees, may represent 'hubs' that many pathogens target to suppress immunity. Another example of the guard model in operation is the indirect recognition of *P. infestans* effector AVR2 by potato R2, via the guarded protein phosphatase BSL1 (Saunders *et al*., 2012). PiAVR2 binds to its host target BSL1, which is detected by R2 and results in a HR (Saunders *et al*., 2012). BSL1 is an ortholog of BSU1 from *Arabidopsis*, which is involved in brassinosteroid (BR) signaling. An over-active BR pathway is known to inhibit PTI responses (Albrecht *et al*., 2012; Belkhadir *et al*., 2012)

and therefore manipulation of this pathway could confer advantages to a pathogen, hence the need for R protein guards.

The decoy model was developed in response to inconsistencies with the guard model and suggests the following: within a host population, where *R* genes are polymorphic, a guarded effector target will be under opposing natural selection pressures. When a functional *R* gene is absent, effector targets with decreased binding affinity to an effector will be selected for, as the target will be less likely to be modified by the effector. However, if a functional R protein is present in a host, evolution will drive the guarded target to increase binding affinity with its effector, making detection by the *R* gene more likely. These considerations put the effector target in an unstable evolutionary position (van der Biezen and Jones, 1998a). The decoy model describes a host protein which acts as a decoy by recognising a pathogen effector, but by having no further role in either disease development or immune response in the absence of a cognate R protein. It is thought that decoys could evolve by gene duplications or through convergent evolution, where a decoy resembles an effector target by evolutionary chance (van der Hoorn and Kamoun, 2008).

1.2.4 Molecular chaperones of NB-LRR proteins

Tight regulation of R proteins by the host is essential to prevent inappropriate signaling and induction of PCD in the absence of a pathogen and to date a small number of R protein-interacting molecular chaperones have been characterised. The most well-known family of molecular chaperones is the heat-shock protein family and one subclass of these proteins, the cytosolic Heat Shock Protein 90 (HSP90) protein family,

plays a particularly important role in plant disease resistance (Hubert *et al*., 2003; Pearl and Promodrou, 2006; Kadota and Shirasu, 2012).

One of the first studies implicating HSP90 in R protein interactions was a genetic screen identifying mutants impaired in Resistance to *Psuedomonas syringae* sp. Maculicola 1 (RPM1)-mediated resistance to the *P. syringae* effector AvrRpm1 (Hubert *et al*., 2003). Single amino acid substitutions in the ATPase domain of HSP90 were found to be responsible for reducing the steady-state levels of RPM1 in non-challenged plants. Subsequent structural analysis of HSP90 revealed a protein with three domains; an N-terminal ATPase domain (ND), a middle domain (MD) implicated in protein binding, and a C-terminal dimerization domain (CD). Dimerisation of two HSP90 proteins has been revealed to be essential for HSP90 function (Pearl and Promodrou, 2006).

The highly conserved SGT1 (Suppressor of G2 allele of SKP1) protein has been found to be an essential component in the regulation of most NB-LRR proteins (Peart *et al*., 2002). It is involved in numerous cellular processes including ubiquitination and kinetochore assembly, as well as the maturation of R proteins (Kadota and Shirasu, 2012). Botër *et al*. (2007) found that accumulation of the potato R protein, Rx, was reliant on the interaction between SGT1 and HSP90. Similar to the structure of HSP90, SGT1 consists of three domains; the tetratricopeptide repeats (TPR), the CHORD (cysteine and histidine-rich domains) -containing protein and SGT1 domain (CS) and the SGT1-specific domain (SGS), with the SGS domain providing the connection between SGT1 and the LRRs of R proteins. Another protein co-chaperone required for R protein regulation is RAR1 (Required for MLA12 Resistance), which is composed of two CHORD domains. CHORD1 and CHORD2 of RAR1 are both zinc-binding, with each

domain binding to two zinc atoms, giving rise to the structure of the protein (Shirasu *et al*., 1999). It is thought that together HSP90, SGT1 and RAR1 form a complex which mediates the stabilisation, maturation and regulation of R proteins.

The HSP90-SGT1-RAR1 ternary complex is thought to be initiated when the CHORD1 domain of RAR1 binds to an ND domain of one HSP90 protein of a HSP90 dimer (Zhang *et al*., 2010). This leads to the CHORD2 domain of RAR1 to be in closer proximity to the ND domain of the second HSP90 protein in the dimer, allowing binding to occur (Zhang *et al*., 2010). The binding of RAR1 to both proteins within the HSP90 dimer, theoretically holds the dimerised proteins in an open conformation allowing the CS domain of SGT1 to bind to the CHORD2 domain of RAR1, thus altering the conformation of SGT1 and boosting its affinity for NB-LRR proteins which are now brought into this protein complex (Kadota and Shirasu, 2012). The binding of RAR1-SGT1-R protein to the HSP90 dimer is thought to allow ATP hydrolysis of the ATP-binding pocket of HSP90. Along with the release of ADP, mature R proteins, SGT1 and RAR1 are able to dissociate from the protein complex (Kadota and Shirasu, 2012).

Whilst binding with HSP90, it is known that SGT1 can at the same time bind with the SKP1P-CDC53P-F BOX (SCF) E3 ubiquitin ligase subunit SKP1 via its TPR (Tetratricopeptide Repeat) domain (Catlett and Kaplan, 2006). This finding has led to proposals that SGT1 could link plant disease resistance with the activity of E3 ubiquitin ligases, suggesting that ubiquitination may play a part in the regulation of NB-LRR proteins (Zhang *et al*., 2010).

1.3 *Phytophthora infestans*

Phythophthora infestans, the oomycete pathogen of potato and tomato, remains as much of a threat to solanaceous crop production as it was in the 18th century when it caused the infamous Irish Potato Famine. Late blight of the Irish potato crop during successive seasons contributed to the death of over a million people and prompted the emigration of another two million people within a decade. Today, *P. infestans* is estimated to cause around £5 billion worth of crop losses globally, equating to 16 % of the potato harvest (Haverkort *et al*., 2009), and particularly severe epidemics can still drive modern farmers out of business (Fry, 2008).

During the last two decades, the global potato industry has seen some major shifts in production trends. Whilst potato production and consumption in developed nations has been in decline since the early 1990s, it has risen significantly in developing countries such as China and India, with these two countries now producing a third of the global potato crop (http://www.fao.org/potato-2008/en/world/). There is now, more than ever a real need to control late blight disease. Fungicides afford some measure of chemical control, but they are financially and environmentally costly, with multiple applications per season required to produce sufficient yields (Vleeshouwers *et al*., 2011). Moreover, fungicide application is only a preventative measure, as once a plant is infected by *P. infestans*, fungicides cannot halt disease progression. A current EU directive (The Pesticides Framework Directive 2009/128/EC) aimed at reducing the usage of pesticides, increases the need for an alternative, low-input late blight-control strategy.

The name "*Phytophthora*" means "plant destroyer" and *Phytophthora infestans* has also earned the moniker "*R* gene destroyer" (Fry, 2008), as it is able to quickly overcome resistances deployed in the field (Colon *et al*., 1995). The emergence and subsequent dominance in recent years of two particularly virulent genotypes of *P. infestans*, A2_13 (Blue 13) and A1_6 (Pink 6) (Cooke *et al*., 2012), highlights the need for an understanding of both host plant resistance and pathogen virulence at a genetic and a molecular level (**Figure 1.2**).

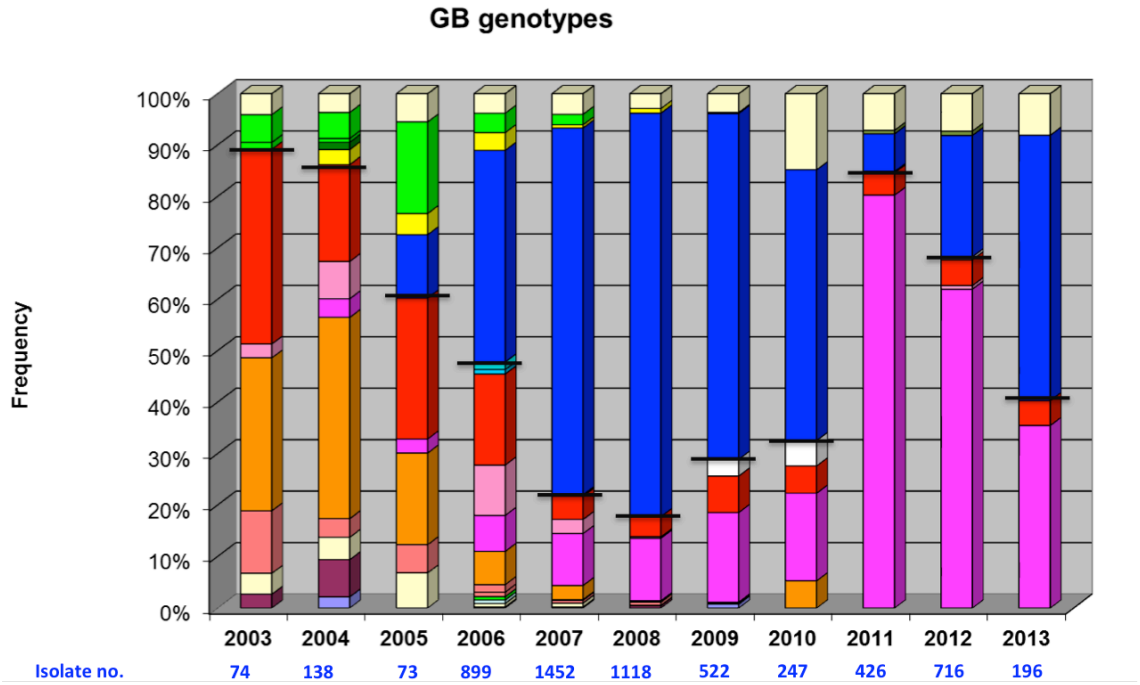

Figure 1.2: The emergence and dominance of the aggressive *P. infestans* isolate A2_Blue13 (kindly provided by Dr. David Cooke). The frequency of multilocus genotypes (MLGs) over the course of 11 years from more than 4000 potato blight outbreaks are shown. The number of isolates fingerprinted each year and dominant MLGs of each mating type are indicated. The blue shaded portions of the bars represent isolate A2_Blue13 and the dark pink sections represent isolate A1_6.

1.3.1 The asexual life cycle of *Phytophthora infestans*

As an oomycete, *P. infestans* remains diploid for the majority of its life cycle during an asexual phase which consists of a biotrophic stage lasting for the first 48 hours of infection and a necrotrophic stage commencing at around 72 hours post-infection. The asexual stage begins with the production of sporangia from sporangiophores, which emerge from infested plant tissues. The sporangia are able to germinate in free water, either through a germ tube under higher temperatures (above 12 ˚C) or by the release of wall-less zoospores under lower temperatures (below 12 ˚C) (Fry, 2008). These zoospores are motile in water as they possess two flagella that aid swimming. The zoospores lose their flagella and rapidly encyst before developing a germ tube which subsequently allows germination (Fry, 2008). The germ tube differentiates into an appressorium which in turn forms a penetration peg able to invade plant cells through the cuticle (Grenville-Briggs *et al*., 2005). Infection vesicles are produced in host epidermal cells and allow the growth of hyphae into the mesophyll layers of the leaf (Grenville-Briggs *et al*., 2005). Haustoria are produced from hyphae, and are presumed to be a means of procuring nutrients from the host (Grenville-Briggs *et al*., 2005) and are important for effector translocation and host colonization (Avrova *et al*., 2008). Under moderate temperatures and humidity, sporangiophores are produced on necrotic lesions, usually on the underside of leaves, after approximately three days, with up to 300, 000 sporangia present in each lesion (**Figure 1.3**) (Grenville-Briggs *et al*., 2005; Fry, 2008).

1.3.2 The sexual life cycle of *Phytophthora infestans*

P. infestans is a heterothallic oomycete and is able to sexually reproduce, as isolates exist in one of two mating types, referred to as either A1 or A2. A1 and A2 mating types are bisexual and can produce either oogonia (female gametes) or antheridia (male gametes) (Judelson, 1997). Mating between an A1 and an A2 type begins with a hormone moving from one mating type to the other and results in the production of haploid antheridia and oogonia, which upon fertilisation, fuse to form diploid oospores (Smoot *et al*., 1958). These oospores may remain in the soil for a number of years due to their thick cell walls. In favourable conditions, oospores germinate and are able to initiate infection in a new host plant (**Figure 1.3**) (Turkensteen *et al*., 2000).

An asexual life cycle ensures that *P. infestans* is able to rapidly reproduce to complete many generations within one growing season, whilst its sexual reproductive strategy allows genetic mixing between isolates. Late blight certainly deserves the name of "Plant Destroyer" as it can decimate previously healthy fields of crops within a matter of days and affects the foliage, stems and tubers of potato plants.

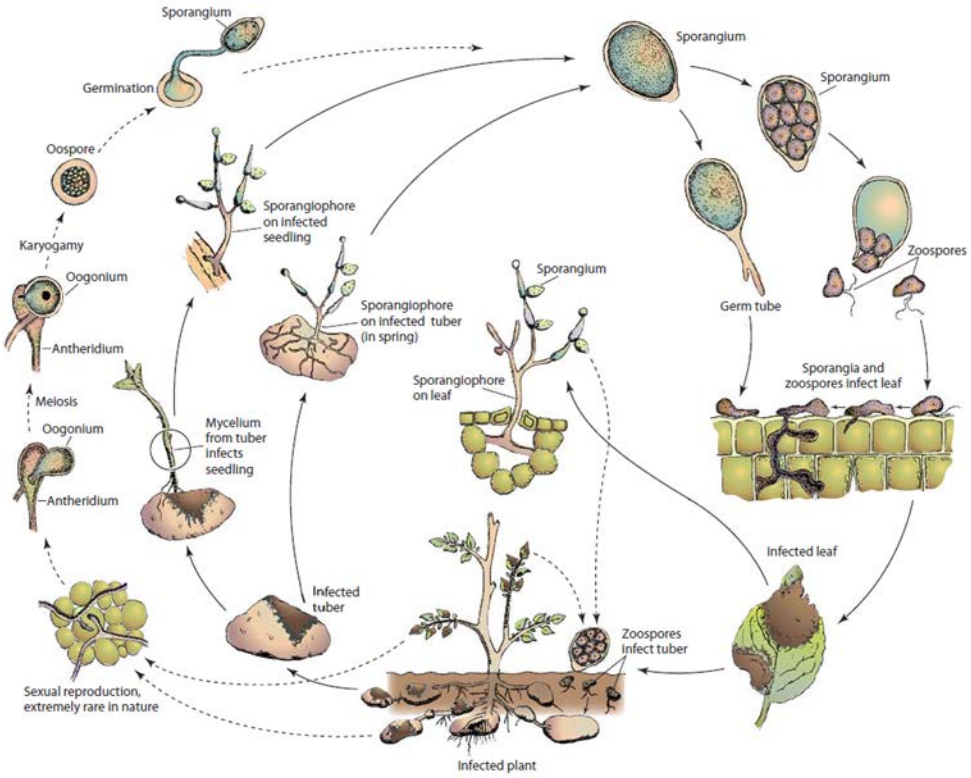

Figure 1.3: The disease cycle of *Phytophthora infestans* on potato (reproduced from Agrios, 2005). The asexual stage of the life cycle begins with the germination of sporangia, either directly or by the release of zoospores which penetrate host leaf tissue to cause disease. The sexual stage of the life cycle occurs when both A1 and A2 mating types are present and results in the production of long-lived oogonia.

1.3.3 The *Phytophthora infestans* genome and its effector complement

The genome sequence of the T30-4 *P. infestans* strain was published in 2009 and revealed a large set of candidate effector proteins available to the pathogen (Haas *et al*., 2009). At least ten genes with AVR activities have been identified in *P. infestans* (Vleeshouwers *et al*., 2011; Rietman *et al*., 2012). These *AVR* genes are all members of the RXLR-EER class of genes, of which there are 563 predicted in the genome of *P. infestans* (Haas *et al*., 2009). See **Table 1.1** for a list of *P. infestans Avr* genes and their corresponding *R* genes from potato. RXLRs are so-called due to the arginine-any amino acid-leucine-arginine motif present in the amino-terminus of the protein. The typical

structure of an RXLR protein follows a conserved pattern; signal peptide, the RXLR domain, often followed by an EER motif, both of which are essential for translocation into the host (Whisson *et al*., 2007), and a diverse, rapidly evolving carboxy-terminal effector domain associated with manipulation of host defences (Win *et al*., 2007). RXLR genes often reside in gene sparse, repeat-rich regions of the *P. infestans* genome, where it is thought they undergo rapid recombination and evolution (Haas *et al*., 2009).

Since the sequenced genome of *P. infestans* was first published, further sequencing projects have revealed the effector complements of other strains of *P. infestans*, including historical genomes preserved in herbaria, the aggressive A2_13 (Blue 13) genotype and closely related sister species of *P. infestans* (Yoshida *et al*., 2013; Cooke *et al*., 2012; Raffaele *et al*., 2010a). Host-adaptation has been identified as the key driver of *P. infestans* genome evolution (Raffaele *et al*., 2010a). By identifying and targeting the most evolutionary stable genes from different *P. infestans* lineages, rational strategies for disease resistance can be developed (Birch *et al*., 2008).

Table 1.1: Identified avirulence (*Avr*) genes of *Phytophthora infestans* and their corresponding *R* genes from potato.

***Avr* gene**	***R* gene**	**References**
Avr1	*R1*	Ballvora *et al*., 2002
Avr2	*R2*	Gilroy *et al*., 2011; Lokossou *et al*., 2009; Saunders *et al*., 2012
Avr3a	*R3a*	Armstrong *et al*., 2005; Bos *et al*., 2010; Engelhardt *et al*., 2012; Huang *et al*., 2005
Avr3b	*R3b*	Jiang *et al*., 2006; Li *et al*., 2011; Rietman 2011; van der Lee *et al*., 2001
Avr4	*R4*	van Poppel *et al*., 2008
Avrblb1	*Rpi-blb1*	Champouret *et al*., 2009; Chen *et al*., 2012; Song *et al*., 2003; van der Vossen *et al*., 2003; Vleeshouwers *et al*., 2008
Avrblb2	*Rpi-blb2*	Bozkurt *et al*., 2011; Oh *et al*., 2009; van der Vossen *et al*., 2005
Avrvnt1	*Rpi-vnt1*	Foster *et al*., 2009; Pel, 2010; Pel *et al*., 2009
AvrSmira1	*Rpi-Smira*	Rietman *et al*., 2012
AvrSmira2/Avr8	*Rpi-Smira2/R8*	Kwang-Ryong, 2013; Kwang-Ryong *et al*., 2011; Rietman *et al*., 2012

1.4 Solanum tuberosum

Potato (*Solanum tuberosum L*.), is a member of the large *Solanaceae* family, which also includes tomato, *Capsicum* species, aubergine, nightshade and tobacco. It is the world's most important non-cereal food crop with production totalling over 376 million tonnes by 2013 (http://faostat3.fao.org). The genome sequence of the doubled monoploid *Solanum tuberosum* Group Phureja (DM) potato was recently published (PGSC, 2011), revealing a predicted 755 NB-LRR genes (Jupe *et al*., 2012; 2013). In contrast to the distribution of RXLRs in the genome of *P. infestans*, potato NB-LRRs reside in genomic regions that are not significantly different to the rest of the potato genome (Jupe *et al*., 2012).

Wild species of potato harbour a rich source of genetic resistance to *P. infestans* and much of this diversity is derived from species from central Mexico. For example, eleven resistance (*R*) genes (*R1*-*R11*) have been identified in the Mexican species *Solanum demissum* and these are represented in the Black and Mastenbroek potato differential set (Black *et al*., 1953, Malcolmson and Black, 1966). This differential set was used for virulence testing of late blight isolates as it was assumed that each differential plant contained a single *R* gene, but it has become clear that most Black and Mastenbroek differential plants harbour multiple *R* genes (Kim *et al*., 2012). The Mastenbroek differential set has recently been updated with *Solanum* sp. plants with reduced *R* gene complexity and with plants containing newly identified *R* genes from different *Solanum* species (Zhu *et al*., 2015). In addition, a separate differential set has been created by genetic modification (GM), with each plant harbouring a single *R* gene in the Desiree genetic background (Zhu *et al*., 2015).

The potato family harbours a rich source of genetic diversity. In addition to the 11 *R* genes identified from *S. demissum*, resistances from other *Solanum* species include *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (van der Vossen *et al*., 2003; 2005; Lokossou *et al*., 2009), *Rpi-pta1* from *S. papita*; (Vleeshouwers *et al*., 2008) and *Rpivnt1.1* from *S. venturii* (Pel *et al*. 2009). See **Table 1.1** for a list of potato *R* genes and their corresponding *Avr* genes from *P. infestans*. Some of these *R* genes have been introgressed into cultivated potato, *Solanum tuberosum*, individually and in combination. However, the highly adaptive *P. infestans* has overcome the majority of deployed resistances. An example of a resistance being rapidly defeated in the field by *P. infestans* is the potato cultivar Pentland Dell, which harbours R1, R2 and R3a. This

cultivar was released in the early 1960s but its resistance was broken within four years of being released in the field (White and Shaw, 2010).

1.5 R3a - AVR3a: a case study of an R protein-effector interaction

One of the best studied examples of an *R* gene-*AVR* gene relationship is that of *R3a* and *AVR3a*, from potato and *P. infestans*, respectively. *R3a,* residing in the *R3* complex locus on the short arm of potato chromosome 11*,* is a member of the CC-NBS-LRR family of *R* genes and was identified using map-based cloning and comparative genomic methods (Huang *et al*., 2005). The *R3a* transcript is 3849 nt long and encodes a predicted polypeptide of 1282 amino acids (a.a.) with a relative molecular mass of 145.9 kDa (Huang *et al*., 2005). *R3a* shares 88% DNA identity and 83% a.a similarity to tomato *I-2*, an *R* gene conferring resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Huang *et al*., 2005). The *R3* complex locus also contains *R3b* which is 82% identical to *R3a* at the nucleotide level and 73% identical at the a.a level, but the two proteins have distinct specificities (Li *et al*., 2011).

R3a recognises the *P. infestans* effector *AVR3a* which exists in two alleles, differing in only two amino acids in the mature protein (Armstrong *et al*., 2005). AVR3a^{KI}, so-called due to the lysine (K) and isoleucine (I) residues at positions 80 and 103 respectively, is recognised in the host cytoplasm by R3a. However, AVR3a^{EM}, containing amino acid residues glutamic acid (E^{80}) and methionine (M^{103}), evades R3a recognition and is regarded as the virulent form of AVR3a (Armstrong *et al*., 2005). Two paralogs of *AVR3a* exist, *Pex147-3* and *Pex147-2* (for *Phytophthora* extracellular protein), the first of which is recognised by R3a whilst the latter is not (Armstrong *et al*., 2005). Western-

blot analysis has shown both AVR3a^{KI} and AVR3a^{EM} are equally stable *in planta* and there is some, weak R3a-dependent recognition of AVR3a^{EM}, when visualised under UV light (Bos *et al*., 2006). In a study using *Agrobacterium*-based co-infiltrations of R3a and AVR3a^{EM} in the model *Solanaceae Nicotiana benthamiana*, macroscopic cell death was observed in 28% of infiltration beginning at 5 days post infiltration (dpi) (Bos *et al*., 2006).

The structure of AVR3a has been elucidated by the structural analysis of close homolog AVR3a4 and AVR1b from *P. sojae*, the pathogen of pepper and curcurbits (Yaeno *et al*., 2011). This study found that a positively charged surface patch of AVR3a binds phosphatidylinositol monophosphates (PIPs) to stabilise the effector protein. Non-PIP-binding AVR3a mutants were significantly less stable than wild-type proteins *in planta*, whilst still being able to activate R3a. The authors suggest that AVR3a binds PIP, allowing the effector protein to accumulate within host cells, from where it can interact with and stabilise CMPG1 (Yaeno *et al*., 2011).

The results from Yaeno *et al*., (2011) are in stark contrast to those found previously which implicate the RXLR domain of AVR1b from *P. sojae* as the site of PIP-binding (Kale *et al*., 2010). The authors suggest that as phosphatidylinositol-3-phosphate (PI3P) is abundant on the outer surface of plant cell plasma membranes, its binding to the RXLR motif allows effector entry into the plant cell (Kale *et al*., 2010). There is much debate over the true site of PIP-binding and further work needs to be carried out, in particular whether the positively charged binding patch identified by Yaeno *et al*., (2011) is required for effector entry into cells (Ellis and Dodds, 2011). Wawra *et al*. (2012) found that amino acids around the RXLR sequence of AVR3a mediate homo-dimerization of the protein and mutation of these residues inhibit this dimerization.

Moreover, the same authors showed that only a denatured AVR3a is able to bind PIP and this interaction is therefore most likely physiologically irrelevant (Wawra *et al*., 2012).

The HR resulting from R3a recognition of AVR3a^{KI} is mediated through the *SGT1* (suppressor of the G2 allele of *skp1*) and *HSP90* (heat shock protein 90) signalling pathway, which is required for the activation of other R proteins (Liu *et al*., 2004; Azevedo *et al*., 2006). Silencing of these genes in *N. benthamiana* abolishes the HR induced by AVR3a^{KI} recognition by R3a and does not affect the stability of the AVR3a protein (Bos *et al*., 2006). The same authors also found that the 75 amino acid C-terminal portion of AVR3a, which excludes the RXLR region, is sufficient for effector activity, which includes the inhibition of cell death induced by the *P. infestans* elicitin INF1 (infestin1) (Bos *et al*., 2006).

The U-box E3 ligase CMPG1, which has been shown to be essential for plant disease resistance (González-Lamothe *et al*., 2006), has been identified as a virulence target of AVR3a (Bos *et al*., 2010). The effector binds to and stabilises the usually transiently expressed CMPG1, with AVR3a^{KI} showing a stronger stabilising effect than AVR3a^{EM}. There is some evidence that CMPG1 is modified by AVR3a-binding as the protein appears as a double band on western-blot analysis (Bos *et al*., 2010). It seems that AVR3a is able to suppress INF1-induced cell death (ICD) by modifying CMPG1 activity, preventing its 26S proteasome-dependent degradation (Bos *et al*., 2010). Deletion and substitution of the terminal tyrosine (Y) of AVR3a^{KI} (AVR3aKI^{Y147del}) has revealed that this residue is not involved in R3a-dependent recognition of AVR3a^{KI} (Bos *et al*., 2009), but is critical for the suppression of ICD and the stabilisation of CMPG1 (Bos *et al*., 2010). It has been found that both the K^{80} and I^{103} residues contribute to the effector

activities of AVR3a^{KI}, as combinations of K^{80}/M^{103} or E^{80}/I^{103} result in a significant loss of cell-death suppression (Bos *et al*., 2006).

Silencing of CMPG1 was found to perturb the HR mediated by Cf-9 recognition of Avr9, Cf-4 recognition of Avr4, and Pto recognition of AvrPto (Gilroy *et al*., 2011b). The PCD induced by the PAMP cellulose-binding elicitor lectin (CBEL) is also CMPG1-dependent. All of these HRs can be suppressed by AVR3a^{KI}, and to a lesser extent by AVR3a^{EM}, but not by the AVR3aKI^{Y147del} mutant. Gilroy *et al*., (2011b) found evidence that the PCD triggered by the cytoplasmic NB-LRR proteins R3a, R2 and Rx was independent of CMPG1 and unaffected by AVR3a, but perception of PAMPs at the inner or outer surfaces of the plasma membrane (PM) is reliant on CMPG1-dependent signal transduction.

Crucially, AVR3a has been shown to be essential for virulence in *P. infestans*, as stable silencing of the effector in *P. infestans* isolate 88069 (CS12) significantly reduced infection on susceptible potato cultivar *Solanum tuberosum* cv. Bintje and on *N. benthamiana* (Bos *et al*., 2010; Vetukuri *et al*., 2011). Complementation experiments where AVR3a^{KI} or AVR3a^{EM} was transiently expressed in *N. benthamiana*, found that both forms of AVR3a restored virulence levels to the *AVR3a*-silenced *P. infestans* strain to those of wt 88069 during the biotrophic phase of infection (Bos *et al*., 2010). No such complementation was observed upon expression of the AVR3a_KI^{Y147del} mutant.

A diversity study of AVR3a in over 80 *P. infestans* isolates from populations collected in the Toluca Valley, Mexico, a centre of potato-*P. infestans* co-evolution, revealed that 72% of isolates were homozygous for *AVR3a^{EM}* (Seman *et al*., in preparation). In contrast, *AVR3a^{KI}* homozygotes accounted for only 2.4% of isolates and were sampled

from wild *Solanum* species exclusively. Other alleles of AVR3a exist at low frequencies, including AVR3aKIL^{139} and AVR3aEMG^{124}, but always in combination with either AVR3a^{KI} or AVR3a^{EM} (Cardenas *et al*., 2011; Seman *et al*., in preparation). AVR3a^{EMG} evades recognition by R3a, whilst AVR3a^{KIL} triggers a HR upon co-infiltration with R3a in *N. benthamiana* (Seman *et al*., in preparation). Both AVR3a^{KIL} and AVR3a^{EMG} are able to stabilise CMPG1 and restore virulence to the AVR3a-silenced *P. infestans* isolate CS12 (Bos *et al*., 2010), however, neither of these alleles were as efficient as AVR3a^{KI} in their ability to stabilise CMPG1 (Seman *et al*., in preparation).

A structure-function analysis utilising saturated mutation of the 88 C-terminal amino acid residues of AVR3a, revealed four R3a-loss-of-function AVR3a^{KI} clones with single amino acid residue mutations (Bos *et al*., 2009). However, it was found that loss-of-recognition of R3a was likely caused by effector protein instability (Bos *et al*., 2009). In an R3a-gain-of-function mutant screen of AVR3a^{EM}, a total of 27 non-redundant single residue mutations were identified, 19 of which were confirmed by agro-infiltration with R3a (Bos *et al*., 2009). Fifteen residues were affected by these mutations, 14 of which were predicted to be surface-exposed residues and all of these mutant proteins were found to be as stable as wild-type proteins *in planta* (Bos *et al*., 2009). Mutation of the K/E^{80} residue of AVR3a to each of the 20 natural amino acids revealed that 14 amino acids at position 80 yielded a HR upon activation by R3a for both AVR3a isoforms (Bos *et al*., 2009). The six mutations which did not cause R3a-gain-of-function, correspond to all amino acids with aromatic (F, Y, W), negatively charged (E, D), or cyclic (P) side chains and may de-stabilise the AVR3a protein (Bos *et al*., 2009).

Despite the high-throughput nature of this study, a mutant AVR3a protein which evades recognition of R3a, yet is able to suppress ICD and stabilise CMPG1, was not

identified. Mutants which were able to evade R3a did not suppress cell death as well as AVR3a^{KI}, which may explain why this isoform is retained at low levels in *P. infestans* as it has exceptional virulence on plants lacking *R3a* (Bos *et al*., 2009).

Upon perception of AVR3a^{KI}, R3a is re-localised from the host cytoplasm to specific, rapidly moving, pre-vacuolar compartments (PVCs) of different size (Engelhardt *et al*., 2012). PVCs are known to be components of the cell endocytic cycle. This re-localisation is not observed upon co-infiltration of R3a and AVR3a^{EM}, which suggests that re-localisation is associated with recognition of the effector. Fluorescently-tagged AVR3a, when infiltrated alone into *N. benthamiana*, is localised in the host cytoplasm, with no evidence for association with vesicles (Bos *et al*., 2010; Gilroy *et al*., 2011b). However, upon recognition by R3a, AVR3a^{KI} is rapidly re-localised to vesicles (Engelhardt *et al*., 2012). The same authors created an auto-active R3a variant, carrying a mutation in the methionine-histidine-aspartate (MHD) motif (D501V) of the NB-ARC domain, which was sufficient to induce a HR upon infiltration in *N. benthamiana* (Engelhardt *et al*., 2012). This auto-activator was localised in the cytoplasm, with and without the presence of AVR3a^{KI}, suggesting that the pathway causing host cell death has been de-coupled from R protein re-localisation (Engelhardt *et al*., 2012).

Yeast-two hybrid (Y2H) analyses yielded no support for direct protein-protein interaction between R3a and AVR3a, but results using bimolecular fluorescence complementation (BiFC), also known as split-YFP assays, have revealed that the two proteins are in close proximity at the PVCs (Engelhardt *et al*., 2012). The negative Y2H experiments do not necessarily rule out a direct interaction between the two proteins, as binding may be transient. Treatment with inhibitors of the endocytic cycle,

Wortmannin and Brefeldin A (BFA), significantly reduced the re-localisation of R3a caused by AVR3a^{KI} after only 30 minutes (Engelhardt *et al*., 2012). These two inhibitors also attenuate the HR normally observed upon co-infiltration of R3a and AVR3a^{KI} in *N. benthamiana*, suggesting that re-localisation is required for a full immune response (Engelhardt *et al*., 2012). The authors speculate that R3a is re-localised to the PVCs so that it can initiate the signalling pathway leading to a downstream immune response (Engelhardt *et al*., 2012).

1.6 PCR shuffling

Also known as directed or accelerated evolution, PCR shuffling was first developed in the early 1990s and has since been used to generate a wide variety of novel genes and proteins (Stemmer, 1994). The process of PCR shuffling is based on iterative rounds of DNA fragmentation, self-priming PCR and selection, requiring only basic molecular methods available in most laboratories. Initially, a parent gene sequence of choice is randomly mutagenized, for example by error-prone PCR, to produce a population of mutant sequences. Alternatively, a number of functional homologous genes can be used. This method, known as family shuffling, uses homologs which are pre-enriched for functional diversity as deleterious mutations have been selected against during the natural process of evolution (Crameri *et al*., 1998). These populations of gene sequences, either mutants or natural homologs, are pooled and randomly fragmented with enzymes or by mechanical processes. The sheared DNA fragments are put into a self-priming PCR, where regions with homology anneal and amplify until a population of shuffled sequences is produced. The resulting shuffled sequences can be cloned into suitable vectors and transformed into an organism of choice, on which a selection can

be applied to identify sequences with enhanced activities. Once identified, the best-performing shuffled sequences can be entered into further iterations of fragmentation, shuffling and selection.

The theory behind PCR shuffling is that naturally occurring genes can be 'improved' when they are shuffled together with functional homologs to give rise to chimeric gene sequences which can encode proteins with beneficial properties. Unlike natural evolution and classical breeding, *in vitro* DNA shuffling can rapidly recombine genes from diverse species. Added to this, mutations thought of as functionally neutral in homologous genes, have proved to be a rich source of diversity and can set the stage for further adaptation *in vitro* (Romero and Arnold, 2009).

1.6.1 PCR shuffling in crop improvement and disease resistance

There are several examples of directed evolution in the field of plant biology, including the PCR shuffling of a glyphosate herbicide tolerance gene which resulted in a mutant gene with enhanced resistance to glyphosate after four iterations (Tian *et al*., 2011). Other work involved shuffling the movement protein (MP) of tobacco mosaic virus (TMV) to enhance the efficiency of replication and movement of the virus in plant hosts (Toth *et al*., 2002). In the area of plant disease resistance, work has been carried out to dissect the functional regions of the tomato resistance gene *Pto* by DNA shuffling (Bernal *et al*., 2005). Pto, a serine/threonine kinase, confers resistance to *Pseudomonas syringae* pv. *tomato* strains expressing the AvrPto or AvrPtoB proteins. Shuffled variants of Pto were generated by PCR shuffling with four functional paralogs and these clones were screened for interaction with AvrPto in yeast. Sequencing of

interacting clones revealed eight candidate regions within Pto associated with binding AvrPto or downstream signalling (Bernal *et al*., 2005).

A combination of PCR shuffled and natural variants of tomato *Cladosporium fulvum* (Cf) resistance genes have been used to investigate auto-active Cf variants which elicit a HR in the absence of fungal elicitors (Wulff *et al*., 2004). Whilst artificially-created *R* gene variants have been used as tools to study plant-pathogen interactions, using PCR shuffling as a technique to enhance the recognition specificity of *R* genes and to create novel resistances is becoming increasingly more important. The potato resistance gene *Rx* is the subject of an on-going effort to fine-tune its recognition specificity to detect additional strains of potato virus *X* (PVX) (Farnham and Baulcombe, 2006; Harris *et al*., 2013). These two studies are discussed in more detail in **Chapter 3** and **Chapter 4**.

The two publications documenting the PCR shuffling of *Rx* show that a stepwise approach to artificial evolution can be used to alter and refine an existing *R* gene whose resistance has been broken in the field (Farnham and Baulcombe, 2006; Harris *et al*., 2013). Another such *R* gene which is an ideal target for PCR shuffling is potato *R3a*, as AVR3a, the essential *P. infestans* effector which is recognised by R3a has low sequence diversity that is conserved in wild populations. Moreover, weak recognition of the virulent AVR3a^{EM} effector form provides the basis for artificial evolution to increase the recognition spectrum of R3a. Two research groups have independently worked on artificially evolving R3a to extend its specificity to recognise both AVR3a forms (Chapman and Stevens *et al*., 2014; Segretin *et al*., 2014). The results from one of these studies are presented in **Chapter 4** (Chapman and Stevens *et al*., 2014).

A pre-requisite before any attempt at artificially evolving an R protein can begin is an in-depth knowledge of the relationship between that protein and its cognate effector molecule. An understanding of the molecular interactions between a pathogen effector and its target, or targets, in the host (previously discussed in **Section 1.2.3**) will give researchers a better idea of which elements of the interaction to target for PCR shuffling. Allelic variants of effectors may interact differently, or not at all, with host-guarded proteins and this should be taken into account before shuffling an R protein. For example, in the case of R2, which detects AVR2 but not AVR2-like 2 (Gilroy *et al*., 2011a), it may be more beneficial to shuffle the virulence target of AVR2, BSL1 (Saunders *et al*., 2012), rather than R2 itself. The aim would be to obtain an evolved guarded host protein that is able to interact with avirulent and previously virulent effector forms in order to mediate detection and an immune response by a host R protein.

1.7 Scope of this thesis

The relationship between potato *R3a* and the *P. infestans* effector *AVR3a* has been the subject of numerous investigations since the two genes were cloned in 2005 and the studies presented in this thesis draw on this extensive body of work. AVR3a presents itself as an ideal target to develop a novel resistance to as it is essential for *P. infestans* pathogenicity, it is conserved in every modern isolate sampled so far and there is limited genetic diversity in wild populations. Although R3a has been defeated in the field, it has an underlying, weak response to $AVR3a^{EM}$, which if enhanced could provide durable resistance to *P. infestans*.

The specific aims of this project were to:

- Determine if non-functional protein homologs and paralogs of wild-type R3a have functional domains.
- Determine the domains of R3a that are involved in effector recognition.
- Characterise gain-of-recognition R3a* variants. Specifically, does the expanded recognition spectrum of these variants translate into durable resistance to *P. infestans* isolates homozygous for $AVR3a^{EM}$?

CHAPTER 2

MATERIALS AND METHODS

2.1 *Phytophthora infestans* cultures

Phytophthora infestans isolates 88069, an *AVR3a*^{EM} homozygote, and 7804.b, an *AVR3a*^{KI} homozygote, were routinely cultured on *N. benthamiana* leaves. Zoospores were collected on a weekly basis by submersion of the leaves in sterile distilled water (sdH_20) and gentle agitation to wash spores from the leaf surface. Spores were inoculated onto fully-expanded leaves from 4- to 6- week-old *N. benthamiana* plants and left to incubate at 18˚C in transparent sealed boxes with 100 % humidity for 7 days. Boxes containing inoculated leaves were kept in darkness for 12 hours post inoculation and then were subjected to the natural day/night cycle.

2.2 Bacterial cultures

All AGL1 strain *Agrobacterium tumefaciens* cultures were grown at 28 °C at 200 rpm for 24 hours in YEB (Yeast Extract Broth), spun at 4000 rpm and the pellet re-suspended in sterile 10 mM 2-(N-morpholine)-ethanesulphonic acid (MES) and 10 mM MgCl2 buffer with 200 µM acetosyringone, to OD_{600} = 0.5 for each construct, unless stated otherwise. Prepared cultures were left to incubate in darkness for 2-4 hours before infiltration into plants. *Escherichia coli* cultures were grown at 28 °C at 200 rpm for 16 hours in LB (Luria Bertani) liquid media. Growth of *R*-gene sequence carrying *E.*

coli cultures at 37 °C was found to result in many gene mutations and deletions, so a temperature of 28 °C was used.

2.3 Plant growth conditions

Plants were grown and maintained throughout the experiments in a glasshouse with a 16 h day period at 22˚C and an 8 h night period at 18˚C. Supplementary lighting was provided below 200 W m^{-2} and screening above 450 W m^{-2}. Agroinfiltration experiments were performed on 4- to 6-week-old *N. benthamiana* plants. Virus-induced gene silencing (VIGS) was performed on *N. benthamiana* seedlings at the 5-leaf stage. Infiltrations for confocal microscopy were performed on 4- to 6- week-old plants.

2.4 Trypan-blue staining of leaves

Before Trypan-blue staining was carried out, leaves were photographed under bright field light. Leaves were incubated in boiling Trypan-blue solution (0.000025 % (w/v) Trypan-blue, 25% (v/v) water, 25% (v/v) phenol, 25% (v/v) lactic acid, 25% (v/v) glycerol) for 5 minutes. After boiling, leaves were washed twice with sterile distilled water, before soaking in saturated chloral hydrate solution (250% (w/v) chloral hydrate in sterile distilled water) overnight at room temperature to remove non-specific staining. A second overnight chloral hydrate soak was carried out and de-stained leaves were photographed over a light box.

2.5 Construction of plasmid vectors

Synthetic constructs pBluescriptIISK.AscI-DM(CC-NBS)-BamHI, pBluescriptIISK.AscI-Pa1(CC-NBS)-BamHI, pBluescriptIISK.BamHI-DM(LRR)-NotI and pBluescriptIISK.BamHI-Pa1(LRR)-NotI were made (Dundee Cell Products, Dundee, UK). The sequence used for DM-Homolog1 (PGSC0003DMG402027402) is available from the additional files supplied by Jupe *et al*., (2012) and the sequence for Paralog1 was taken from the GenBank accession AY849383.1.

DM-CC-NBS and Pa1-CC-NBS fragments from *Asc*I/*Bam*HI digested pBluescriptIISK.AscI-DM(CC-NBS)-BamHI and pBluescriptIISK.AscI-Pa1(CC-NBS)-BamHI, respectively, were ligated into *Asc*I/*Bam*HI digested pGRAB.wtR3a (described previously in Chapman and Stevens *et al*., 2014), resulting in pGRAB.DM(CC-NBS)-R3a(LRR) and pGRAB.Pa1(CC-NBS)-R3a(LRR) constructs (referred to pGRAB.DM-R3a and pGRAB.Pa1-R3a, respectively, hereafter) (**Figure 2.1**).

DM-LRR and Pa1-LRR fragments from *Bam*HI/*Not*I digested pBluescriptIISK.BamHI-DM(LRR)-NotI and pBluescriptIISK.BamHI-Pa1(LRR)-NotI, respectively, were ligated into *Bam*HI/*Not*I digested pGRAB.DM-R3a and pGRAB.Pa1-R3a, respectively (described above), resulting in pGRAB.DM(CC-NBS)-DM(LRR) and pGRAB.Pa1(CC-NBS)-Pa1(LRR) constructs (referred to pGRAB.DM-DM and pGRAB.Pa1-Pa1, respectively, hereafter) (**Figure 2.1**).

DM-LRR and Pa1-LRR fragments from *Bam*HI/*Not*I digested pBluescriptIISK.BamHI-DM(LRR)-NotI and pBluescriptIISK.BamHI-Pa1(LRR)-NotI, respectively, were ligated into *Bam*HI/*Not*I digested pGRAB.wtR3a, resulting in pGRAB.R3a(CC-NBS)-DM(LRR) and

pGRAB.R3a(CC-NBS)-Pa1(LRR) constructs (referred to pGRAB.R3a-DM and pGRAB.R3a-Pa1, respectively, hereafter) (**Figure 2.1**).

DM(CC-NBS)-R3a(LRR) and Pa1(CC-NBS)-R3a(LRR) fragments were generated from pGRAB.DM(CC-NBS)-R3a(LRR) and pGRAB.Pa1(CC-NBS)-R3a(LRR), treated with *Asc*I, T4 DNA polymerase and *Not*I in order. These fragments were ligated into pENTR1a treated with *Sal*I, T4 DNA polymerase and *Not*I in order, to generate pENTR1a.DM-R3a and pENTR1a.Pa1-R3a, respectively. Gateway LR Clonase reactions (Invitrogen, California, USA) between pB7WGY2 (Karimi *et al*., 2005) and either pENTR1a.DM-R3a or pENTR1a.Pa1-R3a generated the YFP-fusions pB7WGY2.DM-R3a and pB7WGY2.Pa1-R3a, respectively.

The plasmid vectors used in the R3a* shuffled variants experiments are described in Engelhardt *et al*. (2012) and Chapman and Stevens *et al*. (2014). See **Table 2.1** for a list of primers used to construct the plasmids detailed in this thesis.

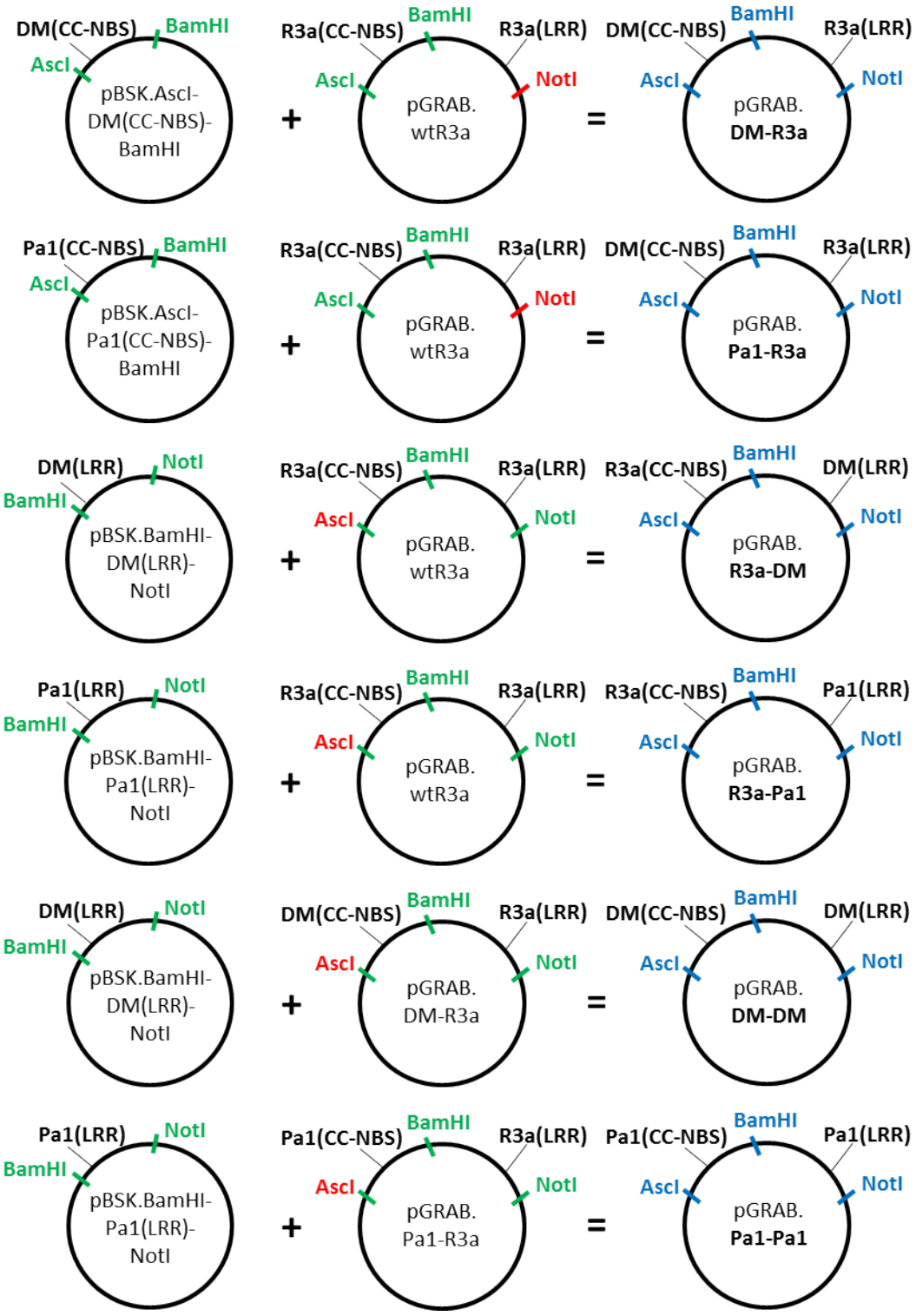

Figure 2.1: Schematic representation of cloning the plasmid constructs for domain swapping experiments. **GREEN** indicates the restriction enzymes used to cut the insert from the donor constructs (left), which were cloned into the vector (middle) once the native insert was removed. **RED** indicates other restriction sites present in the vectors (middle) but not cut in those reactions. **BLUE** indicates the restriction sites present in the newly cloned constructs (right). CC-NBS = Coiled-Coil-Nucleotide Binding Site domain, LRR = Leucine Rich Repeat domain, DM = DM-Homolog1, Pa1 = Paralog1 of R3a, R3a = wt R3a, pBSK = pBluescript II SK(-). *Bam*HI sites between the CC-NBS and LRR domains were silently generated (whilst maintaining the codon coding) and are situated just after the ARC site in the CC-NBS domain.

Table 2.1: Primers used for sequencing the vectors described in this thesis.

Primer name	Purpose	Primer Sequence
DM-NB-SeqPrimF	Sequencing vectors containing the NB domain of the DM homolog	5'-GTCATTCATCTGTGGATTGCC-3'
DM-LRR-SeqPrimF1	Sequencing vectors containing the LRR domain of the DM homolog	5'-GGAGACACCCATCCAACTTTC-3'
DM-LRR-SeqPrimF3	Sequencing vectors containing the LRR domain of the DM homolog	5'-CAATTCCTTGTGATCAGCGC-3'
Par1-LRR-SeqPrF1	Sequencing vectors containing the LRR domain of the Pa1 paralog	5'-GCCTAGACTAAGATCCTTGAGG-3'
Par1-LRR-SeqPrF2	Sequencing vectors containing the LRR domain of the Pa1 paralog	5'-AGTAGTAGTGCCGACAATTCAC-3'
Par1-LRR-SeqPrF3	Sequencing vectors containing the LRR domain of the Pa1 paralog	5'-GAGGGAATGAAGCAGATTGAGG-3'
Par1-LRR-SeqPrF4	Sequencing vectors containing the LRR domain of the Pa1 paralog	5'-TGTGAGAAACTGGTGAATGGACG-3'
pGRAB-F-primer	Sequencing over the 5’ (Forward) junction in pGRAB	5’-AGCATTCTACTTCTATTGCAGCA-3’
pGRAB-R-primer	Sequencing over the 3’ (Reverse) junction in pGRAB	5’-GAGAGAGACTGACGTACGGC-3’
SeqPrimer-1-R	Sequencing vectors containing the NB domain of the Pa1 paralog	5’-GCGACCAATTTGCTTTTCCA-3’
SeqPrimer-2-F	Sequencing vectors containing the NB domain of the Pa1 paralog	5’-TTGCAGAAACAAGCAACCAG-3’
SeqPrimer-2-R	Sequencing vectors containing the NB domain of the Pa1 paralog	5’-TGTCATTCCACACATCATCCA-3’
SeqPrimer-3-F	Sequencing vectors containing the NB domain of the Pa1 paralog	5’-TGGATGATGTGTGGAATGACA-3’
SeqPrimer-3-R	Sequencing vectors containing the NB domain of the Pa1 paralog	5’-TTCATCTTCCTGTGGTATGAGAC-3’
SeqPrimer-4-F	Sequencing vectors containing the Pa1 paralog NB domain	5’-AAGTTATTCATCTGTGGATTGCC-3’
SeqPrimer-4-R	Sequencing vectors containing the wt R3a LRR domain	5’-CCGTTCACCAGTTTCTTGCA-3’
SeqPrimer-5-F	Sequencing vectors containing the Pa1 paralog LRR domain	5’-CCTGAAGGTGGATTGCCC-3’
SeqPrimer-5-R	Sequencing over the 3’ junction in pGRAB	5’-CAACACATGAGCGAAACCCT-3’

2.6 Agroinfiltrations and hypersensitive response (HR) assays

Plasmids pGRAB.DM-DM, pGRAB.Pa1-Pa1, pGRAB.DM-R3a, pGRAB.Pa1-R3a, pGRAB.R3a-DM, pGRAB.R3a-Pa1, pB7WGY2.DM-R3a (YFP and pB7WGY2.Pa1-R3a were transformed in to *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) carrying the helper plasmids pSoup and pBBR1MCS1.VirGN54D (van der Fits *et al*., 2000). All *Agrobacterium* cultures were grown overnight and, after pelleting, re-suspended in sterile infiltration buffer (10 mM MES, 10 mM MgCl2, and 200 µM acetosyringone) to a final $OD_{600\ of}$ 0.3 for confocal microscopy experiments (or as indicated otherwise), 0.5 for western-blot assays or 0.25 for HR assays.

For HR assays, bacterial strains containing the constructs of interest, expressed from the 35S promoter, were mixed, adjusting each strain concentration to a final OD_{600} of 0.25. After an incubation period of 2 to 4 h in darkness at room temperature, bacterial suspensions were infiltrated with a 1 ml blunt-ended syringe through the abaxial leaf surface of 4- to 6-week-old plants, which were superficially wounded with a needle. On each plant, two leaves of each plant were infiltrated, with at least six plants used in each replicate. Bright field/UV pictures were taken 3 and 7 dpi. Development of a HR in infiltrated sites was monitored from 3-7 dpi. A HR was recorded in sites showing necrosis in over 50% of the infiltrated area.

For confocal microscopy experiments, bacterial suspensions containing YFP-fusions of wt R3a, DM-R3a, Pa1-R3a, Rd2-1, Rd3-1 or Rd4-1 were re-suspended to a final OD_{600} of 0.3, $AVR3a^{KI/EM}$ containing suspensions were adjusted to an OD_{600} of 0.1 and suspensions containing the silencing suppressor p19 (Voinnet *et al*., 2003) was re-suspended to an OD_{600} of 0.05. Suspensions containing constructs of interest were

mixed and infiltrated as described previously. Confocal microscopy was carried out 48 hours post-infiltration. Infiltrations for western-blot assays were performed as described for confocal microscopy experiments, but samples were taken and immediately frozen in liquid nitrogen 48 hours post-infiltration.

2.7 *Agrobacterium tumefaciens* transient assays (ATTAs)

Functional *Agrobacterium tumefaciens* transient assays (ATTAs) were carried out in *N. benthamiana*. For the ATTAs described in **Chapter 3**, cultures carrying pGRAB.DM-R3a, pGRAB.Pa1-R3a, pGRAB.wtR3a or pGRAB empty vector were re-suspended as described before to OD_{600} = 0.1 for each construct. Each of the four re-suspensions were infiltrated into separate areas of leaves. Two leaves on each of sixteen plants were infiltrated in each replicate. Two days post infiltration, leaves were detached and infiltration sites inoculated with *AVR3a*^{KI} homozygous *P. infestans* isolate 7804.b. Leaves were incubated in transparent sealed boxes at 100% humidity in a cool room and covered for the first 12 hours. Lesion sizes were measured up to 7 dpi. Co-infiltrations of wild-type R3a, DM-R3a, Pa1-R3a with AVR3a^{KI} and AVR3a^{EM} constructs were carried out contemporaneously in all experiments to confirm that the conditions were conducive to HR development.

For the ATTAs described in **Chapter 4**, cultures carrying pGRAB.-R3a::R3a, pGRAB.R3a::Rd2-1, pGRAB.R3a::Rd3-1, pGRAB.-R3a::Rd4-1 or pGRAB empty vector were re-suspended as described before to OD_{600} = 0.1 for each construct. Each of the five re-suspensions were infiltrated into separate areas of each leaf. Two leaves on each of sixteen plants were infiltrated in each replicate. Two days post infiltration,

leaves were detached and infiltration sites inoculated with *AVR3a*^{KI} homozygous *P. infestans* isolate 7804.b or *AVR3a*^{EM} homozygous isolate 88069. Leaves were incubated in transparent sealed boxes at 100% humidity in a cool room and covered for the first 12 hours. Lesion sizes were measured up to 15 dpi. Co-infiltrations of pGRAB.-R3a::R3a, pGRAB.R3a::Rd2-1, pGRAB.R3a::Rd3-1, pGRAB.-R3a::Rd4-1 with AVR3a^{KI} and AVR3a^{EM} constructs were carried out contemporaneously in all experiments to confirm that the conditions were conducive to HR development.

2.8 Confocal laser scanning microscopy

Imaging was performed on a Leica TCS-SP2 AOBS microscope (Leica Microsystems) using HCX APO L, 40x/0.8, and 63x/0.9 water dipping lenses or a Zeiss 710 using a Plan APO 40x/1.0 water dipping lens. Images were collected using line by line sequential scanning. The optimal pinhole diameter and the same gain levels were used within experiments. YFP and CFP were imaged using 514 nm and 405 nm excitation, respectively, and emissions were collected between 520-563 nm and 455-490 nm, respectively. Photoshop CS5.1 software (Adobe Systems) was used for post-acquisition image processing. Whilst the experimental design and set-up of plant material to be imaged in **Chapter 4** was performed by me, images were taken by Dr. Petra Boevink on the Zeiss 710 confocal microscope, due to a limit on the number of people who could be trained on this microscope.

2.9 Western-blot assays

Protein extraction from yeast cells was performed as described by Kushnirov (2000). Extraction of total protein from plant samples was done by grinding leaf tissue in liquid nitrogen followed by boiling for 5 min in SDS loading buffer supplemented with 1 % β-mercaptoethanol. Samples were spun at 13, 000 rpm for 5 min to separate the soluble proteins from the insoluble pellet. The presence of recombinant wtR3a, DM-R3a, Pa1-R3a, Rd2-1, Rd3-1 or Rd4-1 fusion proteins was determined by SDS-PAGE and protein gel blotting as described previously (Engelhardt *et al*., 2012). For the detection of YFP fusions, an α-GFP from rabbit was used (Sigma-Aldrich, Santa Cruz Biotechnology); followed by α-rabbit-horseradish peroxidase from goat (Sigma-Aldrich). Protein bands on the immunoblot were detected using ECL substrate (GE Healthcare). Western-blot experiments were repeated at least three times. All western-blot experiments were performed with the help of Dr. Stefan Engelhardt.

2.10 Virus-induced gene silencing (VIGS) of *SGT1* and *HSP90*

Tobacco rattle virus (TRV)-induced gene silencing in *N. benthamiana* was performed as described previously (Bos *et al*., 2006). *Agrobacterium* cultures transformed with the binary TRV RNA1 construct, pBINTRA6, or the TRV RNA2 vector constructs PTV00, PTV:eGFP, PTV:HSP90 or PTV:SGT1 were re-suspended to OD_{600} = 0.5 for the RNA1 construct and OD_{600} = 1.0 for the RNA2 constructs. Re-suspended RNA1 and RNA2 cultures were mixed in a 1:1 ratio and infiltrated into noncotyledonous leaves of *N. benthamiana* plants at the 5-leaf stage. For each of the biological replicates, six plants per treatment were used and six plants were used as non-TRV controls. Three weeks

after treatment with the VIGS constructs, plants were infiltrated with culture mixtures (OD_{600} = 0.5) designed to express R3a, Rd2-1, Rd3-1 or Rd4-1 and $AVR3a^{KI}$ or $AVR3a^{EM}$. HRs were scored at 6 dpi and photographs of leaves under bright field and UV light were taken at this timepoint.

2.11 Sequence analysis

Unless otherwise stated, all sequence analysis was performed using the BioEdit software package.

2.12 Statistical analysis

All statistical analysis was performed in R version 3.0.3 and Minitab ® 17. Statistical analysis on all data was performed using one-way ANOVA. All statistical tests run in R and Minitab indicated approximate normality and equality of variance for all of the data.

CHAPTER 3

DOMAIN SWAPPING BETWEEN WILD-TYPE R3a AND ITS HOMOLOGS AND PARALOGS

3.1 INTRODUCTION

Plant disease resistance is a multi-layered process of host surveillance of invading microbial pathogens. The first layer of inducible responses involves the detection of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) which are perceived by pattern recognition receptors (PRRs) in the host, resulting in pattern-triggered immunity (PTI). PTI is suppressed by adapted pathogens which deploy effector molecules to perturb host processes, creating more amenable conditions for pathogen infection. Effector proteins are detected by host resistance (R) proteins which form the second layer of plant inducible defences. The largest class of R proteins identified to date is the nucleotide binding, leucine-rich repeat (NB-LRR) protein family (McHale *et al*., 2006).

The NB-LRR family can be divided into two groups depending on the domain present at the N-terminus of the protein. TIR-NB-LRRs (TNLs) have N-terminal domains with homology to the Drosophila toll and human interleukin-1 receptor (TIR), whilst some CC-NB-LRRs (CNLs), have a predicted coiled-coil (CC) domain within the protein's N-terminus (McHale *et al*., 2006). NB-LRRs are modular proteins made up of well-defined domains. Within the extended NB domain, that itself has a number of well-defined motifs (Jupe *et al*., 2012), resides a large interaction complex referred to as ARC named after the first three proteins it was identified in; human apoptotic protease-activating

factor-1 (APAF-1), plant R proteins and *Caenorhabditis elegans* death-4 protein (CED-4) (van der Biezen and Jones, 1998b). Proteins carrying an NB-ARC domain belong to the STAND (signal transduction ATPases with numerous domains) family of NTPases (Lukasik and Takken, 2009). The C-terminal LRR domain of NB-LRR resistance proteins has long been associated with pathogen recognition specificity and it is thought that the difference in numbers of leucine-rich repeats and the appearance of irregular motifs within this domain have evolved in response to continuous pathogen surveillance (Ellis *et al*., 1999; Farnham and Baulcombe, 2006; Ellis *et al*., 2007).

Resistance proteins are thought to operate as molecular switches and have distinct states: an autoinhibited "off" state and an active "on" state which coincides with pathogen detection and activation (Lukasik & Takken, 2009). The NB-ARC domain functions as a nucleotide binding pocket as ADP is bound in the closed conformational "off" state but is exchanged for ATP resulting in the "on" state (Tameling *et al*., 2006). Through interactions with the NB-ARC domain, the LRR domain is known to contribute to protein stability and auto-inhibition (Qi *et al*., 2012; Slootweg *et al*., 2013).

The potato resistance protein R3a, a member of the CNL family (Huang *et al*., 2005), recognises the essential effector AVR3a from *Phytophthora infestans* in a well-characterised molecular plant-pathogen interaction (Armstrong *et al*., 2005; Engelhardt *et al*., 2012). One form of the effector, $AVR3a^{KI}$, is recognised *in planta* by R3a, leading to a hypersensitive response (HR), whilst another form, $AVR3a^{EM}$, which differs in only two amino acids in the mature effector protein, evades this recognition (Armstrong *et al*., 2005).

Located on the long arm of potato chromosome 11 within the large R3 locus, R3a confers race-specific resistance to *P. infestans* isolates containing the avirulent AVR3a^{KI} form of the effector (Huang *et al*., 2005). In a recent study, the mechanism behind the R3a-mediated recognition of AVR3a was elucidated (Engelhardt *et al*., 2012). Upon recognition of AVR3a^{KI}, but not the AVR3a^{EM} effector form, both AVR3a^{KI} and R3a rapidly re-localise from the host cytoplasm to late endosomes, components of the endocytic pathway, which is thought to be a prerequisite for subsequent HR development (Engelhardt *et al*., 2012).

Initially cloned in 2005, R3a occurs in a cluster with three closely related paralogous genes (Huang *et al*., 2005). The authors showed that when each of these four genes were transformed separately into susceptible potato clone 1029-31, only the gene later designated as R3a was capable of conferring resistance to *P. infestans* isolates 89148-9 and IPO-0, which both harbour AVR3a^{KI} (**Figure 3.1**; Huang *et al*., 2005). Paralogous genes occur by duplication within a genome and are able to evolve new functions, which can be related to the function of the original gene. The functions of the three paralogs of R3a are currently unknown.

Although, the sequenced doubled monoploid potato *Solanum phureja* clone DM1-3 516 R44 (DM) has no functional resistance to current late blight isolates tested (**Figure 3.1**), highly related homologs of the cloned potato late blight resistance genes *R1*, *RB*, *R2*, *R3a*, *R3b*, *Rpi-blb2* and *Rpi-vnt1.1* have been found in the genome of DM (The Potato Genome Sequencing Consortium [PGSC], 2011). There are 755 NB-LRR genes in DM (Jupe *et al*., 2012; 2013), many of which are non-functional pseudogenes due to indels, frameshift mutations and premature stop codons (PGSC, 2011). Homologs are defined as genes which are related by descent from a common ancestral DNA

sequence, so homologs could also be referred to as orthologs if the genes were separated by a speciation event. Orthologs usually retain the original function of the ancestral gene in each new species.

A BLAST analysis of DM NB-LRRs (Jupe *et al*., 2013) alongside previously published R3a sequences (Huang *et al*., 2005) has identified 46 sequences with homology to the CC-NB domains of R3a. The phylogenetic relationship of the deduced NB amino acid sequences was established with the help of Dr. Katrin Mackenzie from BioSS (**Figure 3.2**). The sequence most similar to R3a is R3a-Paralog 1 (AY849383.1), which was previously identified by Huang *et al*. (2005). *R3a* and three of its paralogs were cloned from the F_1 population of SH83-92-488 (SH) $_{x}$ RH89-039-16 (RH), two different genotypes of *S. tuberosum* (Huang *et al*., 2005). However, the R3 cluster was initially introgressed from *S. demissum*, the 'donor' species of most characterized race-specific *R* genes to *P. infestans* (Huang *et al*., 2004). The CC-NB domains of R3a and R3a-Paralog1 share 96 % sequence similarity at the amino acid level (**Figure 3.3**). The sequence from DM with the highest similarity to R3a is a non-functional homolog (DMG402027402) which seemingly has a truncated LRR, although its CC-NB domain shares 93 % homology at the amino acid level with the CC-NB domain of R3a (**Figure 3.3**).

A

B

Construct	Clone	No. plants tested[a]	No. plants showing resistance to *P. infestans* isolate[b] 89148-09	IPO-0	H30P04
*I*2GA-SH23-1	1029-31	12	0	0	0
	Desiree	6	0	0	0
*I*2GA-SH23-2 (*R3a*)	1029-31	8	8	8	0
	Desiree	7	7	7	0
*I*2GA-SH23-3	1029-31	13	0	0	0
	Desiree	5	0	0	0
*I*2GA-SH194-2	1029-31	23	0	0	0
	Desiree	8	0	0	0
Control	1029-31	10	0	0	0
	Desiree	10	0	0	0
	RH	10	0	0	0
	SW8540-025	10	10	10	0
	SH	10	10	10	10

Figure 3.1: Wild-type R3a paralogs and homologs do not confer resistance to AVR3a-carrying blight isolates. (**A**) Various blight isolates inoculated onto the leaves of the sequenced DM potato clone were able to cause disease, including the AVR3a^{KI}-carrying 7804.b isolate. The image was taken 7 days after blight inoculation. (**B**) Disease test of primary transformants of R3a (I2GA-SH23-2) and its three paralogs, I2GA-SH23-1 (Paralog1), I2GA-SH23-3 and I2GA-SH194-2. Numbers of plants showing resistance to the three *Phytophthora infestans* isolates IPO-0, H30P04, and 89148-09 are indicated. 89148-09 and IPO-0 carry AVR3a^{KI}, whilst H30P04 is homozygous for AVR3a^{EM}. Table taken from Huang *et al*. (2005).

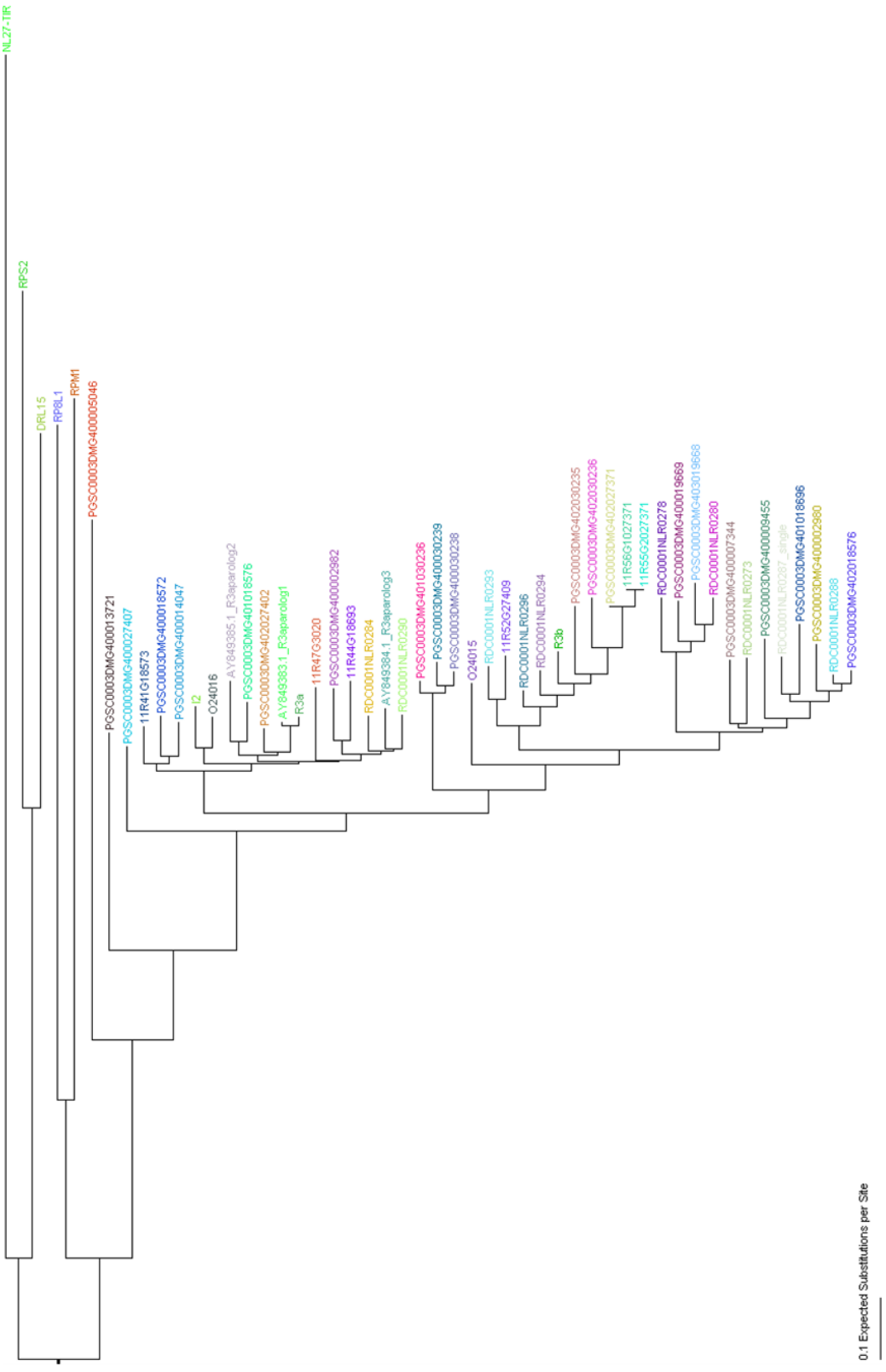

Figure 3.2: Phylogenetic analysis of the R3 family including *R3a* as well as *R3b* homologs and paralogs and *I2*. Sequences of the NB-ARC domains of predicted R3a homologs and paralogs were used, alongside selected NB-ARC domains from functional resistance genes, to study the phylogenetic relationships between them. The different colours of the sequence names are for ease of reading. Constructed with the help of Dr. Katrin MacKenzie.

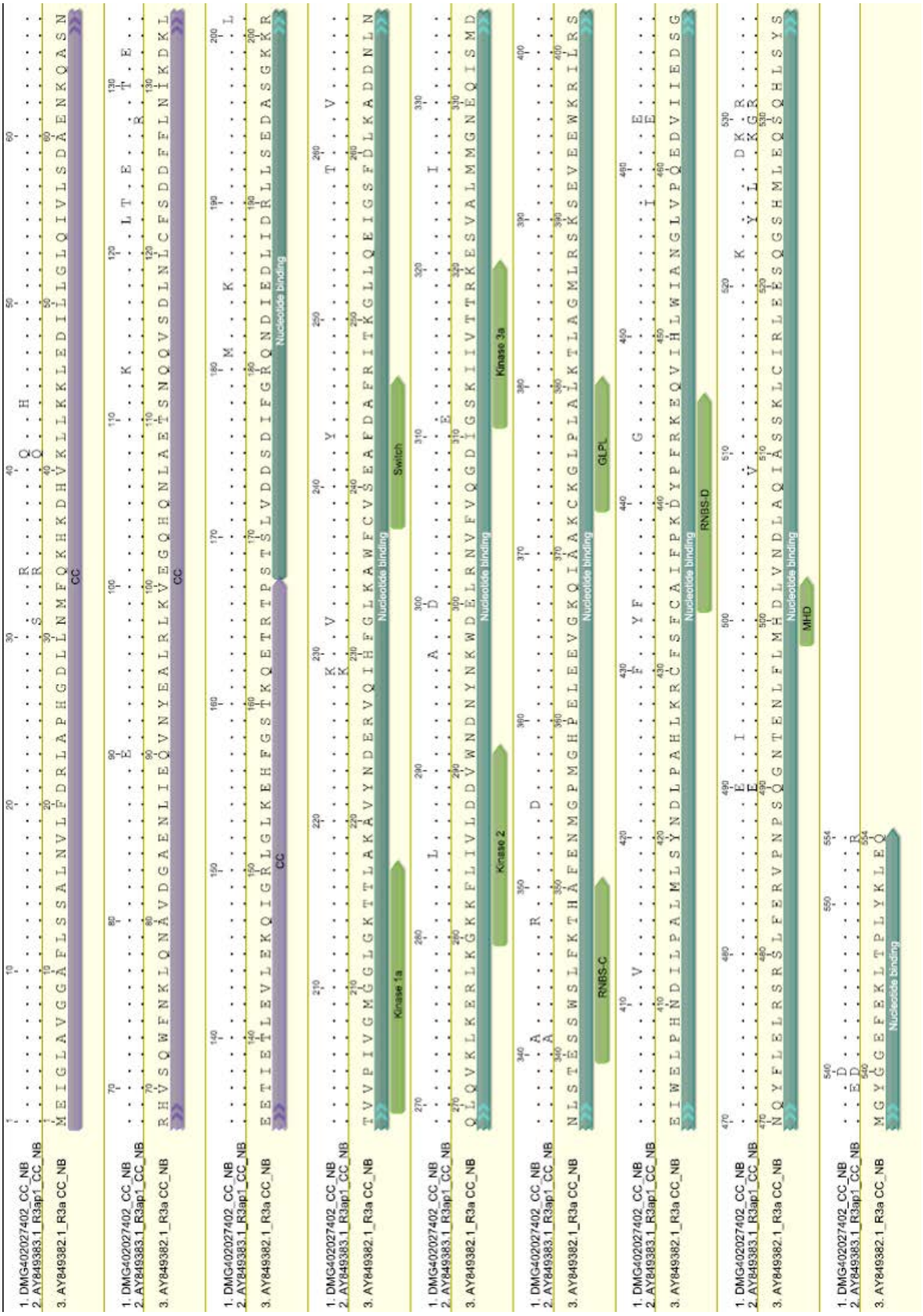

Figure 3.3: Alignment of amino acid sequences comparing the CC-NB domains of paralogs and homologs of R3a. Amino acid sequences of wild-type R3a (AY849382.1), the DM homolog DMG402027402 and the R3a-Paralog-1 AY849383.1 were aligned and visualised using the Geneious package, Biomatters Limited.

3.2 AIMS

In this study, domain swapping experiments were carried out to identify key domains required for R3a function. For this I assessed whether specific domains from non-functional R3a homologs and paralogs can be cloned in frame with wild-type R3a domains to result in functional chimeric proteins. The aims of the experiments detailed in this chapter were:

a) To investigate the recognition specificity of wild-type proteins DMG402027402 (referred to as DM) and R3a-Paralog 1 (AY849383.1 – referred to as Pa1).
b) To investigate the recognition specificity of chimeric proteins created between the LRR domain of wild-type R3a and the CC-NB domains of its paralog and a homolog, R3a-Paralog 1 and DMG402027402, respectively.
c) To investigate the recognition specificity of chimeric proteins created between the CC-NB of wild-type R3a and the LRR domains of its paralog and a homolog, R3a-Paralog 1 and DMG402027402, respectively.
d) To determine whether chimeric constructs provide resistance to the $AVR3a^{KI}$ homozygous strain of *Phytophthora infestans*, 7804.b.
e) To investigate the cellular localisation of the chimeric proteins in comparison to wild-type R3a when expressed alone and in the presence of recognised and unrecognised effector proteins.
f) To determine whether chimeric proteins are stably expressed *in planta.*

3.3 RESULTS

3.3.1 Wild-type DM and Pa1 proteins do not recognise AVR3a

The two genes, DM and Pa1, with the highest sequence similarity to R3a were cloned into the 35S driven binary expression vector pGRAB (**Figure 3.4**). The recognition spectra of these proteins were tested in three independent biological replicates of transient co-infiltration assays in *N. benthamiana*. Neither the wild-type DM or Pa1 proteins elicited a HR upon co-infiltration with either form of AVR3a, or when expressed in the absence of the effector (**Figure 3.5**). A one-way ANOVA performed on the data from the three independent biological replicates showed that recognition of AVR3a by both the wild-type DM and Pa1 proteins was significantly different to R3a-mediated recognition of AVR3a ($F_{8,27}$ = 108.25, p < 0.001, n = 30, **Figure 3.5**). When viewed under UV light, there were no indications of auto-fluorescence associated with host cell death caused by the accumulation of phenolic compounds, in sites infiltrated with the native genes DM-R3a or Pa1-R3a (**Figure 3.5**).

3.3.2 Recognition specificities of chimeric proteins DM-R3a and Pa1-R3a indicate that the LRR domain of R3a is involved in effector recognition

The recognition spectra of DM-R3a and Pa1-R3a chimeric proteins (see **Figure 3.4**) were assessed in four independent biological replicates of transient co-infiltration assays in *N. benthamiana*. The Pa1-R3a chimera showed a similar recognition spectrum to that of the wild-type R3a protein, as there was strong recognition of $AVR3a^{KI}$ but weak recognition of $AVR3a^{EM}$ (**Figure 3.5**). The DM-R3a chimera did not elicit a HR in the presence of either form of AVR3a. However, when imaged under UV light, weak

auto-fluorescent phenolic compounds associated with host cell death were seen in leaf areas co-infiltrated with DM-R3a and AVR3a^{KI}, indicating a weak recognition of this effector form by the chimeric protein (**Figure 3.5**). However, a t-test comparing the percentage HR values for wild-type R3a expressed in the absence of an effector and DM-R3a in the presence of AVR3a^{KI}, showed no significant difference between these two values ($p = 0.41$). Neither the DM-R3a nor Pa1-R3a chimeras showed signs of auto-activity when expressed in the absence of the effector (**Figure 3.5**). A one-way ANOVA performed on the data from the four independent biological replicates showed that there was no significant difference between the wild-type R3a- and Pa1-R3a-mediated recognition of AVR3a^{KI} ($F_{8,27} = 54$, $p < 0.001$, n = 170, **Figure 3.5**).

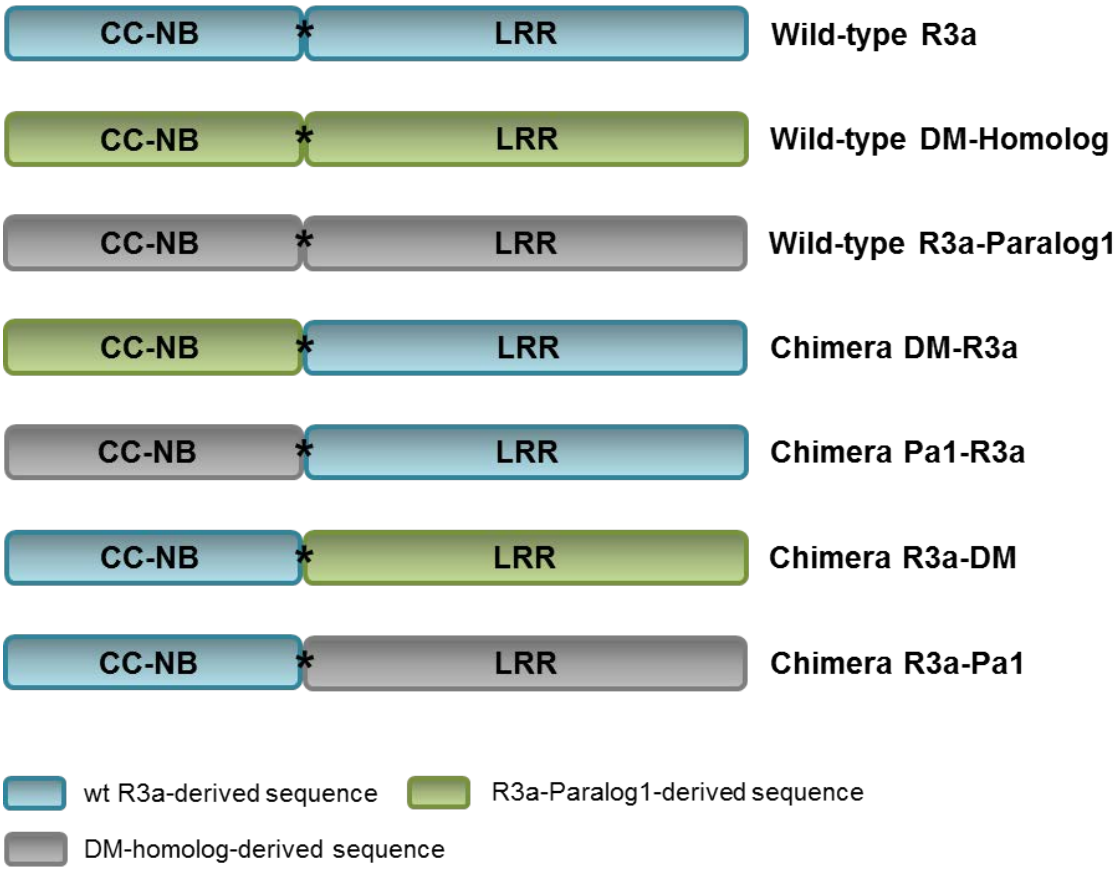

Figure 3.4: Schematic representation of the constructs created for domain swapping experiments. CC-NB = Coiled-Coil-Nucleotide Binding site domain, LRR = Leucine Rich Repeat domain, * = indicates the position where a *Bam*HI restriction site was silently generated (maintaining the codon coding) in order to aid the cloning of these constructs. The *Bam*HI sites are situated just after the ARC domain within the CC-NB domain.

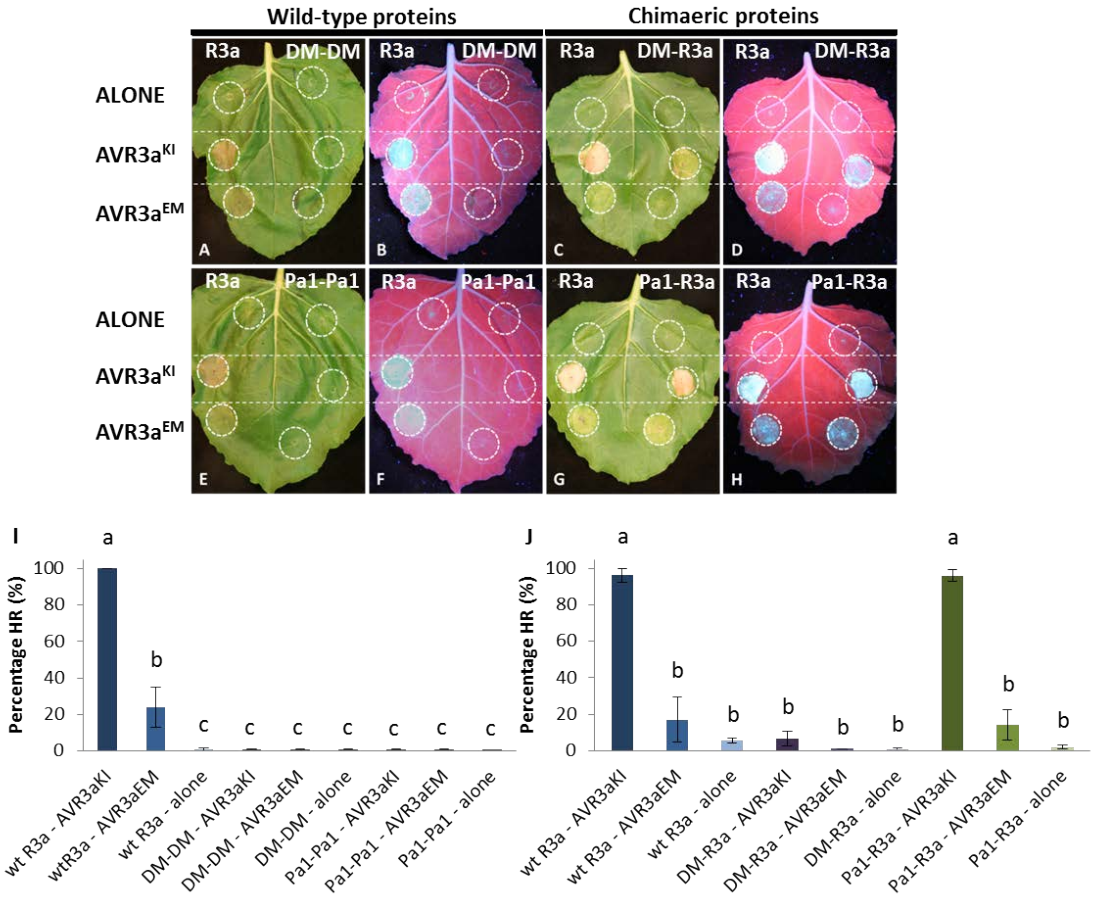

Figure 3.5: Recognition spectra of wild-type and chimeric R3a/R3a-like proteins. Indicated constructs were transiently co-expressed by agro-infiltration in *N. benthamiana*. Images were taken 3 days after agro-infiltration under white light (**A**, **C**, **E** and **G**) and UV-B (**B**, **D**, **F** and **H**). Circles indicate the infiltrated areas on the leaf panels. (**I**) Mean percentage HRs of R3a-like proteins measured 3 days after agro-infiltration from three independent biological experiments. Shared letters above the bars indicate that those means are not significantly different, grouping information generated with the Tukey Method (95 %), error bars indicate ± SEM, zero values have been transformed to 1% to facilitate their observation, n = 30, $F_{8,27}$ = 108.25, $p < 0.001$. (**J**) Mean percentage HRs of chimeric R3a (LRR) proteins measured 3 days after agro-infiltration from four independent biological experiments. Shared letters above the bars indicate that those means are not significantly different, grouping information generated with the Tukey Method (95 %), error bars indicate ± SEM, zero values have been transformed to 1% to facilitate their observation, n = 170, $F_{8,27}$ = 54, $p < 0.001$.

3.3.3 Chimeric proteins R3a-DM and R3a-Pa1 do not recognise AVR3a

Chimeric proteins R3a-DM and R3a-Pa1 (**Figure 3.4**) did not elicit any HR upon co-infiltration in *N. benthamiana* with either form of AVR3a, or when expressed in the absence of the effector (**Figure 3.6**). A one-way ANOVA performed on the data from three independent biological replicates showed that recognition of AVR3a by both the R3a-DM and R3a-Pa1 chimeric proteins was significantly different to R3a-mediated recognition of AVR3a ($F_{8,27}$ = 108.25, p = 0.000, n = 30, **Figure 3.6**). When viewed under UV light, no auto-fluorescence was observed in sites infiltrated with either R3a-DM or R3a-Pa1 (**Figure 3.6**).

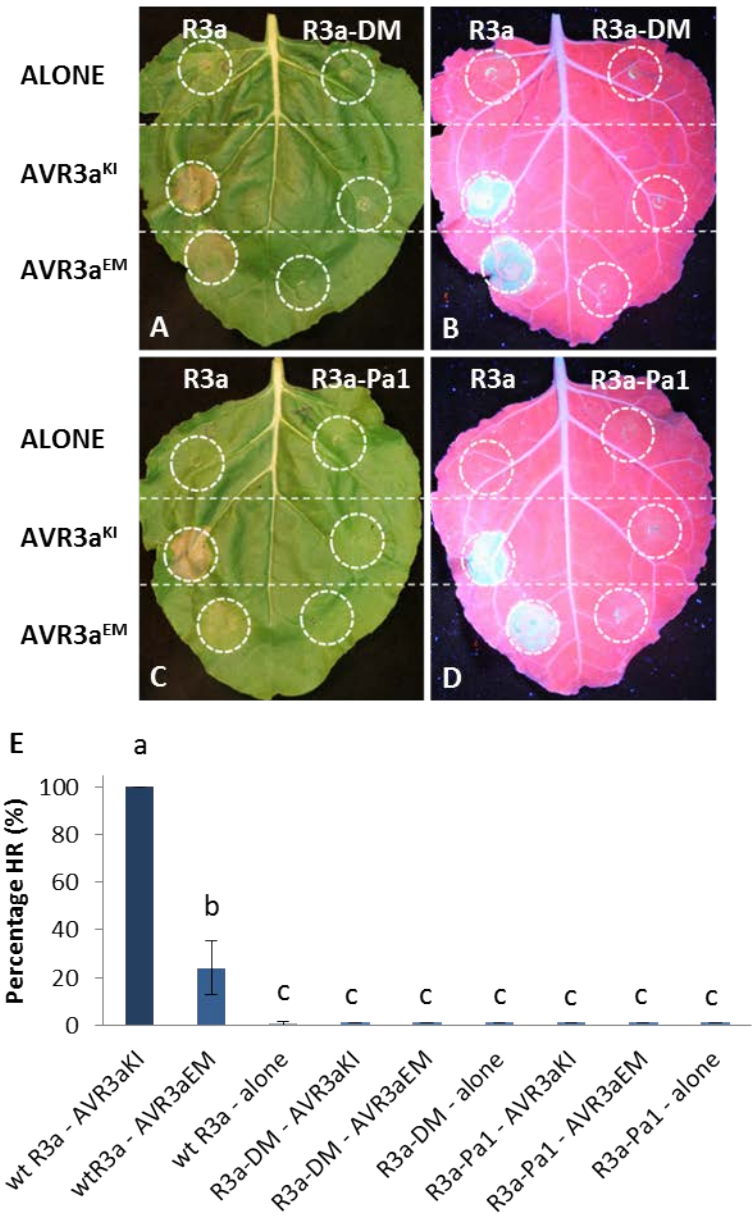

Figure 3.6: Chimeric proteins with the CC-NB domain of wt R3a do not recognise AVR3a. Constructs were transiently co-expressed by agro-infiltration in *N. benthamiana*. (**A-D**) Control infiltrations of wt R3a with AVR3a^{KI}, AVR3a^{EM} or in the absence of an effector are shown on the left side of each leaf panel and confirm that conditions were conducive for HR development. Images were taken 3 days after agro-infiltration under white light (**A** and **C**) and UV-B (**B** and **D**). Circles indicate the infiltrated areas on the leaf panels. (**E**) Mean percentage HRs of chimeric R3a (NB) proteins measured 3 days after agro-infiltration from three independent biological replicates. Shared letters above the bars indicate that those means are not significantly different, grouping information generated with the Tukey Method (95 %), error bars indicate ± SEM, zero values have been transformed to 1% to facilitate their observation, n = 30, $F_{8,27} = 108.25$, $p < 0.001$.

3.3.4 The Pa1-R3a chimera provides resistance to *P. infestans* isolate 7804.b

Agrobacterium tumefaciens transient assays (ATTAs) were carried out to compare resistance between the DM-R3a and Pa1-R3a chimeras, wild-type R3a and an empty vector control. A one-way ANOVA performed on the data from three independent biological replicates showed that constructs had significantly different effects on *P. infestans* growth ($F_{3,8}$ = 7.84, p = 0.009, n = 94, **Figure 3.7**). Wild-type R3a and the Pa1-R3a chimera significantly reduced the spread of *P. infestans* compared to the empty vector control (**Figure 3.7**). This result indicates that the recognition of $AVR3a^{KI}$ by Pa1-R3a (**Figure 3.5**) translates into functional resistance to an $AVR3a^{KI}$ homozygous *P. infestans* isolate. There was no statistically significant difference between the resistance provided by wild-type R3a and the Pa1-R3a chimera (**Figure 3.7**). The DM-R3a chimera consistently reduced the spread of *P. infestans* relative to the empty vector control, although this effect was non-significant (**Figure 3.7**). A t-test comparing the mean lesion diameter values for the empty vector control and the DM-R3a chimera, showed no statistically significant difference (p = 0.29).

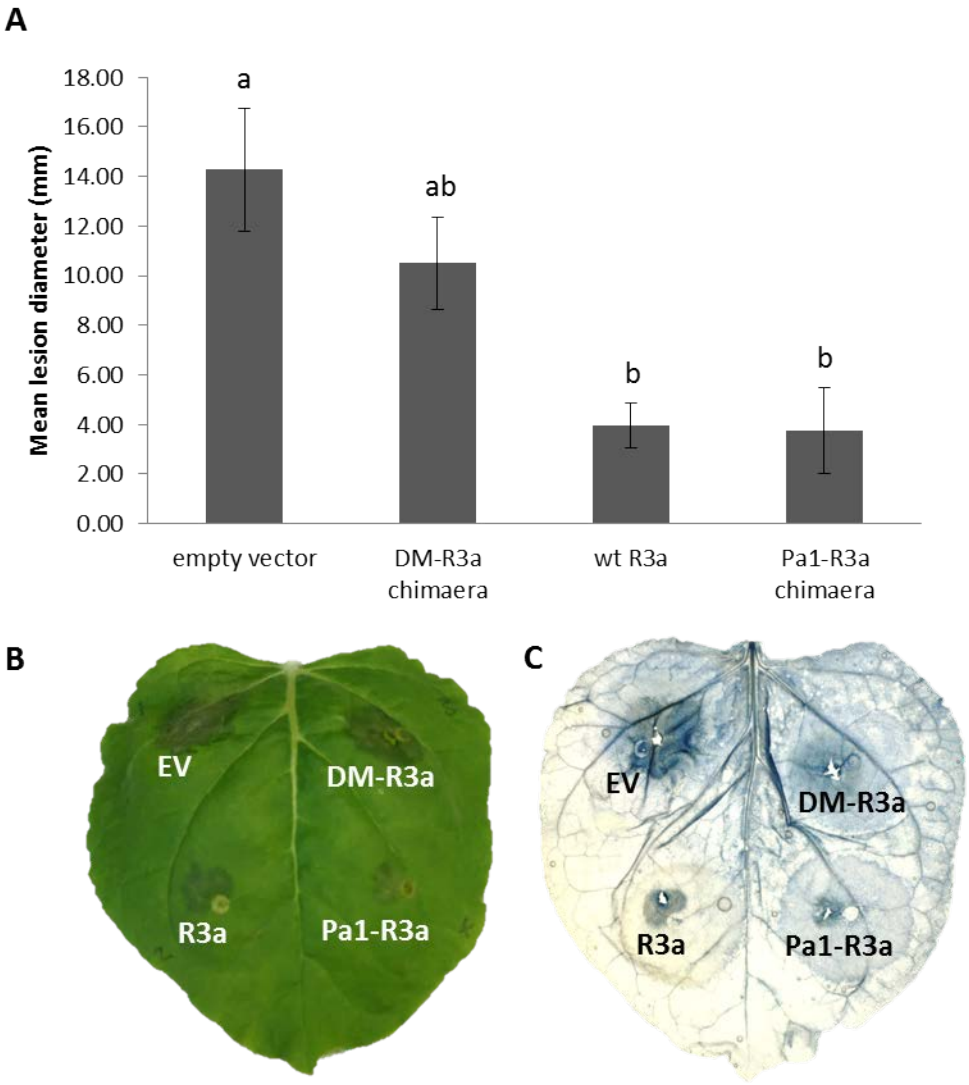

Figure 3.7: R3a and chimeric protein Pa1-R3a transiently expressed in *N. benthamiana* from *Agrobacterium* reduce the spread of AVR3a^{KI}-expressing *P. infestans* strain 7804.b. (**A**) Mean lesion diameters measured 7 days after drop inoculation of agro-infiltrated areas with strain 7804.b (KI/KI) from three independent biological replicates. Shared letters above the bars indicate that those means are not significantly different, grouping information generated with the Tukey Method (95 %), error bars indicate ± SE_M, n = 94, $F_{3,8}$ = 7.84, p = 0.009. (**B** and **C**) Leaves showing disease progression of 7804.b on the agro-infiltrated sites indicated, under white light (**B**) and after trypan blue staining (**C**). EV = empty vector control.

3.3.5 The Pa1-R3a chimera re-localises to vesicles upon co-infiltration with AVR3a^{KI} but the DM-R3a chimera remains cytoplasmic in the presence of AVR3a

To determine the localisation pattern of chimeric proteins DM-R3a and Pa1-R3a, N-terminal fusions of DM-R3a and Pa1-R3a with yellow fluorescent protein (YFP) were generated with expression of these constructs driven by a 35S promoter.

Following transient expression in *N. benthamiana*, YFP-R3a (Engelhardt *et al*., 2012) and YFP-chimera protein fusions YFP-DM-R3a and YFP-Pa1-R3a displayed cytoplasmic localisations in the absence of the AVR3a effector (**Figure 3.8**). In accordance with Engelhardt *et al*. (2012), the localisation of YFP-R3a remained cytoplasmic upon co-infiltration with AVR3a^{EM}, but re-localised to fast moving vesicles, following co-infiltration with AVR3a^{KI}. The YFP-DM-R3a fusion protein, which when in its untagged form does not strongly recognise either form of AVR3a, remained cytoplasmic in the presence of both AVR3a^{KI} and AVR3a^{EM} (**Figure 3.8**). However, the YFP-Pa1-R3a fusion protein, which shows recognition of AVR3a^{KI}, re-localised to fast-moving vesicles upon co-infiltration with AVR3a^{KI} (**Figure 3.8**). As expected, in the presence of unrecognised AVR3a^{EM}, YFP-Pa1-R3a remained cytoplasmic. As demonstrated for YFP-wild-type R3a fusions by Engelhardt *et al*. (2012), YFP-chimeric fusions did not elicit HRs alone or in the presence of AVR3a^{KI} or AVR3a^{EM}, probably due to steric hindrance of the signalling domains of the chimeric proteins (**Figure 3.8**). Three independent biological replicates of the confocal microscopy assays were carried out, with the same phenotypes consistently identified in each of the three replicates.

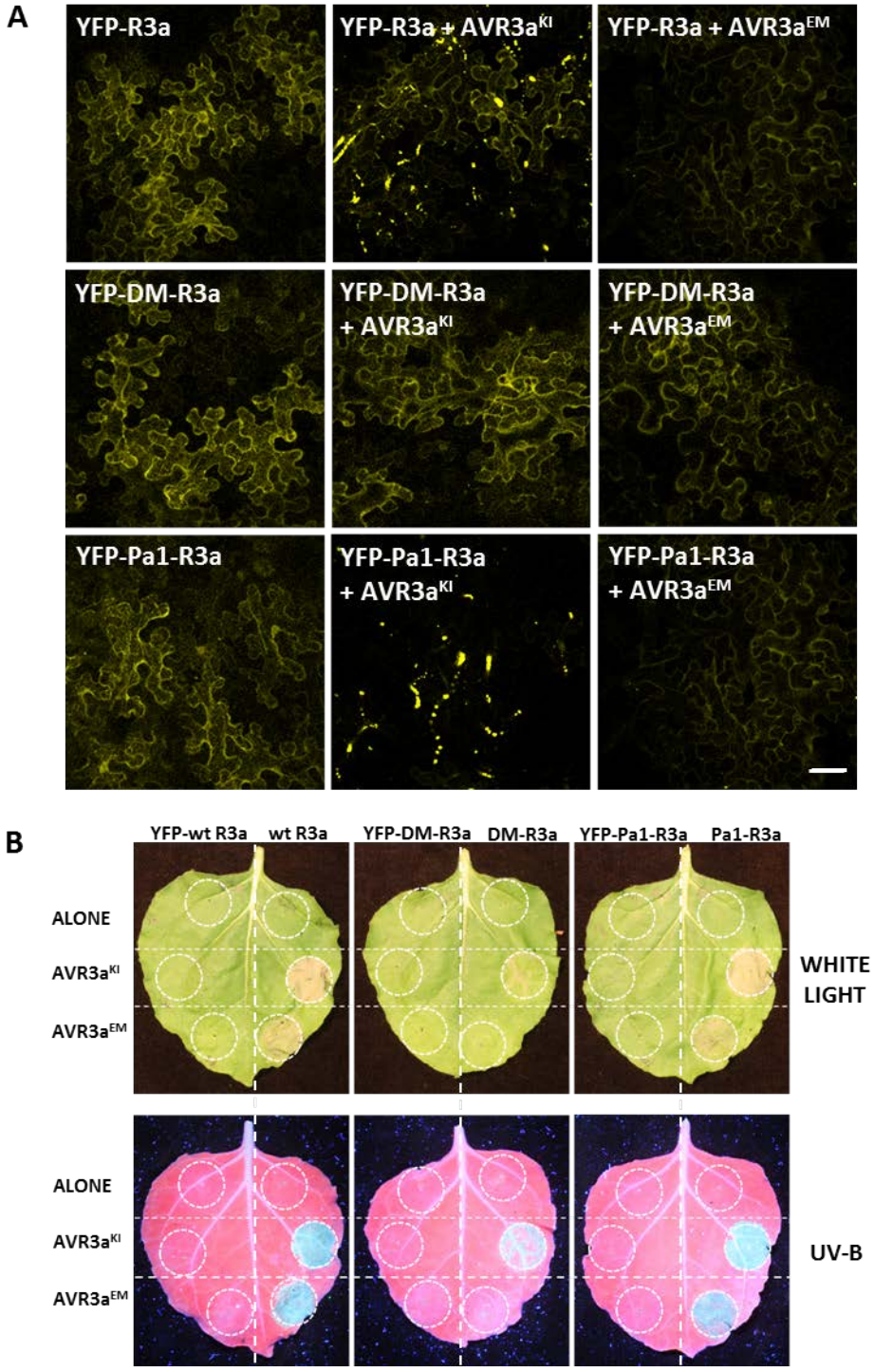

Figure 3.8: Cellular localisation of chimeric R3a/R3a-like proteins. (**A**) YFP- fused wt R3a and the Pa1-R3a chimera re-localise to vesicles upon perception of AVR3a^{KI}. YFP-DM-R3a remains cytoplasmic in the presence of AVR3a^{KI} and AVR3a^{EM}. YFP-R3a, YFP-Pa1-R3a and YFP-DM-R3a are cytoplasmic in the absence of the effector and in the presence of AVR3a^{EM}. Two days after infiltration of mixtures of *Agrobacterium tumefaciens* cultures expressing AVR3a^{KI}, AVR3a^{EM}, YFP-R3a, YFP-Pa1-R3a or YFP-DM-Pa1 fusions, *N. benthamiana* leaves were examined under a confocal laser scanning microscope. Representative images are from three independent biological replicates. Scale bar = 50 µm. (**B**) YFP C-terminally fused wt R3a, DM-R3a and Pa1-R3a prevents HR development. Constructs were transiently co-expressed by agro-infiltration in *N. benthamiana*. Images were taken 3 days after agro-infiltration under white light (upper) and UV-B (lower). Circles indicate infiltrated areas.

3.3.6 The Pa1-R3a chimera is stable when expressed *in planta*

To assess if YFP-DM-R3a and YFP-Pa1-R3a are stable *in planta*, western-blot analysis of protein extracts from inoculated *N. benthamiana* tissue was carried out. A single replicate of the western-blot experiment showed that whilst the Pa1-R3a is stably expressed *in planta*, the DM-R3a chimera appears to be potentially less stable as this protein was not detected in this assay (**Figure 3.9**). This western-blot experiment seemed to show that in the presence of the recognised form of the effector, AVR3a^{KI}, both wild-type R3a and the Pa1-R3a chimera were detectable at lower levels in the soluble protein extraction fraction than when expressed alone or in the presence of the unrecognised AVR3a^{EM} (**Figure 3.9**).

In addition, the untagged wild-type R3a and Pa1-R3a constructs provide resistance to AVR3a^{KI}-carrying blight isolates in detached leaf assays, suggesting that they are expressed at high enough levels to confer resistance.

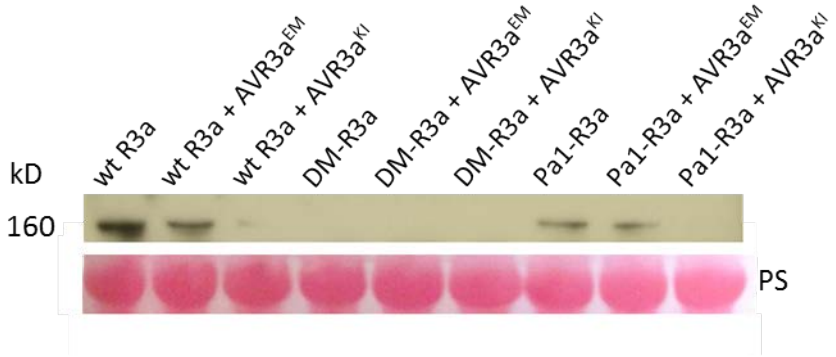

Figure 3.9: Stability of chimeric R3a/R3a-like proteins. Immunoblot probed with α-GFP following transient expression of wild-type YFP-R3a, YFP-Pa1-R3a and YFP-DM-R3a alone or co-expressed with either AVR3a^{EM} or AVR3a^{KI} in *N. benthamiana* at 2 d after inoculation. Protein sizes are indicated (in kilodaltons, kD), and protein loading is shown by Ponceau stain (PS).

3.4 DISCUSSION

Work done by Jupe *et al*., (2012; 2013) to identify *R* gene sequences in the sequenced DM potato genome has detected 26 DM homologs of *R3a* and *R3b* which form the phylogenetic subgroup CNL-8. Twenty-four of these homologs have been physically mapped to chromosome 11 (Jupe *et al*., 2012). However, none of these DM *R3a* homologs provide functional resistance to AVR3a^{KI}-carrying strains of *P. infestans* (**Figure 3.1**), whilst the three paralogs identified alongside R3a are also non-functional (Huang *et al*., 2005). This study aimed to elucidate whether R protein chimeras made from different domains of functional and non-functional sequences could recapitulate the recognition and resistance provided by wild-type R3a and to identify key domains that are required for maintaining R3a function. The initial analysis to identify closely related homologs and paralogs of R3a included newly identified NB-LRRs characterised by the re-annotation of the DM genome by Jupe *et al*. (2013), as well as selected NB-ARC domains from functional resistance genes (**Figure 3.2**).

Domain swapping experiments conducted here have shown that the CC-NB domain from the previously non-functional R3a-Paralog1 (I2GA-SH23-1) protein can be fused to the native LRR of R3a, resulting in successful recognition and resistance to effector AVR3a^{KI} by the Pa1-R3a chimera (**Figures 3.5** and **3.7**). However, the CC-NB domain from the more distantly related DMG402027402 R3a-homolog is unable to reconstitute this level of recognition and resistance in the DM-R3a chimera (**Figures 3.5** and **3.7**).

Weak recognition provided by the DM-R3a chimeric protein is reminiscent of the low level of recognition exhibited by wild-type R3a in the presence of the AVR3a^{EM} effector

form (**Figure 3.5**) (Bos *et al*., 2006). In fact, weak recognition of the virulent effector form has been abolished in the DM-R3a chimera, whereas the Pa1-R3a chimera displays weak recognition of AVR3a^{EM} whilst retaining strong recognition of AVR3a^{KI}: the same pattern observed for wild-type R3a (**Figure 3.5**).

The weak but visible DM-R3a-mediated recognition of AVR3a^{KI} may explain the weak resistance conferred by this chimeric protein to an AVR3a^{KI} homozygous strain of *P. infestans*. Three independent replicates of a detached leaf ATTA experiment consistently showed that the DM-R3a chimera reduces the spread of disease caused by an AVR3a^{KI} homozygous strain of *P. infestans* when compared to an empty vector control, although this was not statistically significant (p = 0.29, **Figure 3.7**). Thus, although not detectable in western-blot analyses (**Figure 3.9**), evidence suggests that there is some DM-R3a chimeric protein expressed. To test whether the DM-R3a chimera does have an effect on the spread of disease, future ATTAs could include the wild-type DM protein. The Pa1-R3a chimera confers a similar level of resistance to strain 7804.b as wild-type R3a, with these values being significantly different to that of the empty vector control (**Figure 3.7**).

Previously, Engelhardt *et al*. (2012) demonstrated that an N-terminally YFP-fused wild-type R3a rapidly re-localises from a cytoplasmic cellular localisation to pre-vacuolar compartments (PVCs), also known as late endosomes, in the presence of the recognised form of AVR3a. This re-localisation was found to be a pre-requisite for subsequent HR development for untagged R3a co-expressed with AVR3a^{KI} (Engelhardt *et al*., 2012). In accordance with these results, I have shown that recognition of AVR3a^{KI} by the Pa1-R3a chimera is accompanied by a re-localisation to fast-moving vesicles (**Figure 3.8**). A YFP-fused DM-R3a chimera remains cytoplasmic in the presence

of either form of AVR3a, reiterating the finding that re-localisation to vesicles does not occur where there is a lack of recognition of the effector (**Figure 3.8**).

One of the three western-blot experiment replicates showed that the Pa1-R3a chimera is stably expressed *in planta*, whilst the DM-R3a chimera is potentially not or is significantly less stable (**Figure 3.9**). The results from this experiment indicate further that the presence of the recognised effector form, AVR3a^{KI}, reduces the amount of detectable protein of both wild-type R3a and the Pa1-R3a chimera, compared to when they were expressed alone or in the presence of unrecognised AVR3a^{EM} (**Figure 3.9**). There are two potential explanations for this observed pattern, the first being that wild-type R3a and the Pa1-R3a chimera are unstable in the presence of AVR3a^{KI}. However, this seems unlikely as Engelhardt *et al*. (2012) reported that wild-type R3a is stable in the presence of AVR3a^{KI}.

The second and more plausible explanation for the observed pattern is that in the presence of the recognised effector, the R protein is re-localised to late endosomes which become bound in the insoluble pellet fraction during protein extraction. One way to test this theory would be to assess the predicted increase of YFP-tagged R proteins in the insoluble protein fraction via a western-blot analysis. A further two replicates of the western-blot experiment did not yield any specific bands for any of the constructs tested, so further experiments are needed.

Our results show that whilst the CC-NB domain of R3a-Paralog1 has remained effective in combination with a functioning LRR domain, the same cannot be said for the LRR domains of homologs and paralogs of R3a. Reciprocal experiments where the LRR domains of the R3a-Paralog1 or the DM-homolog were fused to the CC-NB domain of

wild-type R3a did not result in functional chimeric R proteins (**Figure 3.6**). The CC-NB domains of R3a-Paralog1 and DM-homolog are 96% and 93% similar to wild-type R3a at the amino acid level, respectively, whilst the LRR domains of both the homolog and the paralog are highly divergent to wild-type R3a's LRR (**Figure 3.3**). The fact that the LRR domains of both the wild-type DM and Pa1 proteins are highly divergent to the LRR domain of wild-type R3a is probably the reason why the DM and Pa1 wild-type proteins do not recognise $AVR3a^{KI}$ (**Figure 3.5**). The LRR domain is thought to be involved in effector recognition, either directly or by recognising host-binding targets of effectors (Ellis *et al*., 1999; Farnham and Baulcombe, 2006; Ellis *et al*., 2007). The divergent amino acid sequences in the two wild-type proteins could have abolished recognition of $AVR3a^{KI}$ or its binding partners in the host.

These results are in line with previous studies on the evolution of plant *R* genes, as it is generally agreed that purifying selection acts on the NB domain, preventing the accumulation of non-synonymous mutations and leading to higher homology levels between related NB domains (Mondragón-Palomino *et al*., 2002; Chen *et al*., 2010). Conversely, high levels of non-synonymous to synonymous nucleotide substitutions suggest that positive diversifying selection maintains variation within the LRR domain (Mc Hale *et al*., 2006; Jacob *et al*., 2013). Compared to R3a, there are similar levels of synonymous to non-synonymous polymorphisms for both the DM-Homolog and R3a-Paralog1 (30 synonymous vs. 33 non-synonymous in the CC-NB domain of the DM-Homolog, and 10 synonymous vs. 11 non-synonymous in the CC-NB domain of R3a-Paralog1). There are 30 synonymous mutations vs. 32 non-synonymous mutations between the CC-NB domains of DM-Homolog and R3a-Paralog1.

In wild plant populations, two models of *R* gene evolution and preservation have been described. The first form is known as an evolutionary 'arms race' where selective sweeps remove old or broken *R* genes from plant populations and new variants become rapidly established (Bergelson *et al*., 2001). 'Trench warfare' describes the balancing selection that retains allelic diversity of *R* genes within populations and gives rise to long-lived *R* gene families (Stahl *et al*., 1999).

Although the DM homolog of R3a is not functional, it has been retained in the genome (along with other supposedly non-functional *R* gene homologs) by balancing selection, and has survived evolutionary sweeps. This suggests that these *R* genes or *R* gene families may have unknown functions, possibly in providing resistance to other pathogens (Schulze-Lefert and Panstruga, 2011). Indeed, in addition to *R3a*, functional *R3b* (Li *et al*., 2011) and *I2* (Ori *et al*., 1997; Simons *et al*., 1998) have arisen from within this gene family.

The sequencing and interrogation of an individual genome provides us with a blueprint of the genetic diversity present within that genome. Although the monoploid DM genome appears not to have any functional *R* genes towards the late blight pathogen *P. infestans*, a wealth of diversity exists within the non-functional sequences which reside in the genome. It is the genetic diversity present within the wider potato population that has allowed R3a to evolve through gene duplication and diversification (Huang *et al*., 2005). This study has shown that paralogs of R3a with functional domains have evolved but only in conjunction with R3a itself. Conversely, although balancing selection has been the driver of the evolution of the R3a family, closely related sequences in DM do not contain functional domains for AVR3a^{KI} recognition. This suggests that the development of functional NB-LRRs requires concerted

evolution of the individual domains. It is worthwhile noting, however, that this study exchanged large domains encompassing CC-NB-ARC coding sequences as well as entire LRRs. It is therefore conceivable that exchange of smaller domains (e.g. CC, NB or ARCs) would have been able to further delimit the critical functional structures of DM.

Indeed, a mutation and domain swapping analysis between potato Rx and Gpa2, immune receptors which recognize potato virus *X* and the potato cyst nematode *Globodera pallida*, respectively, found that co-evolution between the different domains of NB-LRR proteins is essential for appropriate recognition function (Slootweg *et al*., 2013). A minimal region of the ARC2 domain and N-terminal LRRs are involved in the activation state of the protein and two amino acid residues in the ARC2 are able to distinguish between auto-activation and effector-triggered activation (Slootweg *et al*., 2013). The results of the study led the authors to propose a new mechanistic model which illustrates how the ARC2, NB, and N-terminal half of the LRR form a clamp, holding the protein in a tightly regulated steady-state formation. Slootweg *et al*. (2013) hypothesise that the tightly controlled structures of R proteins, achieved through inter-domain physical interactions, impose constraints on the evolution of new resistances, as only fine-tuned cooperation between domains results in functional R proteins. The authors concluded that swapping random sequences between homologous *R* genes readily leads to non-functional or auto-active NB-LRR proteins (Slootweg *et al*., 2013).

In a separate study of the potato Rx protein, Rairdan and Moffet (2006) had previously demonstrated that the ARC1 sub-domain (within the CC-NB domain) of Rx is required for physical interaction between the N terminus of the protein and the LRR domain. In addition, a deletion analysis of the LRR domain of Rx revealed that the entire LRR domain is required for binding to the Rx CC-NB-ARC fragment (Rairdan and Moffett,

2006). The authors highlight the possibility that there may be multiple contact points between the N- and C-termini of the protein in order to maintain tight interaction between domains (Rairdan and Moffett, 2006). Domain swapping experiments identified the ARC2 sub-domain as playing a role in regulating Rx's transition from an inactive to an active state, as mutations and disruptions within this region resulted in a constitutively active protein (Rairdan and Moffett, 2006).

In the *Arabidopsis* RPS2 protein, which mediates resistance to *Pseudomonas syringae* pathovars expressing the effector *avrRpt2*, the LRR domain was shown to be the key determinant of allelic resistance or susceptibility (Banerjee *et al*., 2001). The authors showed that plants of the *Arabidopsis* Col-0 ecotype were resistant to *P. syringae* expressing *avrRpt2*, whilst plants of the Po-1 ecotype were susceptible to the same bacteria (Banerjee *et al*., 2001). However, the *RPS2* allele present in *Arabidopsis* ecotype Po-1 can function in a Col-0 genetic background, but not in a Po-1 background. Other host resistance-determining genes present in Po-1 confer resistance in combination with the RPS2 allele from Col-0 (Banerjee *et al*., 2001). Domain swap experiments between the amino- and LRR domains of Col-0 and Po-1 *RPS2* alleles revealed the difference in interaction between Col-0 and Po-1 *RPS2* and other host loci is caused by a polymorphism of six amino acids in the LRR domain (Banerjee *et al*., 2001).

Fine domain swapping experiments between the non-NB-LRR Cf-9 and Cf-9B proteins from tomato has revealed the specific amino acid residues in central LRRs involved in recognition of the Avr9 protein from the leaf mould fungus *Cladosporium fulvum* (Chakrabarti *et al*., 2009). Both proteins confer resistance to *C. fulvum*, but only Cf-9 confers seedling resistance and recognizes Avr9 (Panter *et al*., 2002). The domain

swapping study found that nine amino acid differences over three consecutive LRRs were sufficient for Cf-9-mediated resistance, whilst most of the LRR domain was required for Cf-9B-specific resistance (Chakrabarti *et al*., 2009). Two of the chimeric proteins created for this study gave differing results in necrosis assays when tested in both *N. benthamiana* and tomato, leading the authors to speculate that the two assays identify unrelated ligands or detect related ligands in slightly different ways (Chakrabarti *et al*., 2009).

Whilst most studies on R protein-effector coevolution have focussed on the nucleotide or amino acid sequences of the plant host proteins, one study has interrogated the natural variation present in alleles of a single effector in order to dissect the evolutionary mechanisms at work. The hypervariable *Arabidopsis* resistance gene *RPP13* mediates resistance to the ATR13 effector from the oomycete pathogen *Hyaloperonospora arabidopsidis* (formerly *Hyaloperonspora parasitica*) (Allen *et al*., 2004). The variability present in the LRR domain of RPP13 is matched by the variation between ATR13 alleles, which could be suggestive of diversifying selection driving the coevolution between the receptor and its effector (Allen *et al*., 2004). Sequence analysis, domain swapping experiments and site-directed mutagenesis of 15 different alleles of *ATR13* revealed that the C-terminus of the effector determines *RPP13*-dependent recognition and identified three key amino acid residues involved in recognition (Allen *et al*., 2008).

Domain swapping experiments using functional protein sequences have proven to be useful tools in identifying the domains and residues involved in effector recognition and resistance. Interestingly, the functional *R* gene most similar to *R3a* is the tomato *I2* resistance gene to *Fusarium oxysporum*, with the two proteins being more related to

one another than other known R proteins, including R3b (Simons *et al*., 1998; Huang *et al*., 2005). R3a and I2 share 83 % similarity at the amino acid level, with considerable dissimilarity occurring at the CC domain, relative conservation at the NB domain and divergence at the LRR domain (Huang *et al*., 2005). However, it appears that the two proteins mediate resistance in very different ways. Whilst R3a re-localises from the cytoplasm to late endosomes upon perception of $AVR3a^{KI}$, (Engelhardt *et al*., 2012), I2 is thought to contain a potential nuclear localisation signal in its CC domain and the nuclear localisation of its cognate effector, FsAvr2, is required to trigger an I2-dependent cell death response (Simons *et al*., 1998; Ma *et al*., 2013). It would be interesting to carry out domain swapping experiments between these two resistance proteins, creating chimeras and testing their recognition and resistance to AVR3a and *Fusarium oxysporum* f sp *lycopersici*.

3.5 CONCLUSIONS

The following conclusions can be drawn from the experiments detailed in this chapter:

a) The native protein products of DMG402027402 and R3a-Paralog 1 (AY849383.1) are not able to recognise AVR3a.

b) Domain swapping experiments to combine the CC-NB domain of closely related paralogs and homologs of R3a with the wild-type R3a LRR domain have shown that the Pa1-R3a chimeric protein is able to recognise AVR3a^{KI}, whilst the DM-R3a chimeric protein cannot.

c) Reciprocal experiments to combine the CC-NB domain of wild-type R3a with the LRR domain of closely related homologs and paralogs showed that neither the R3a-DM or R3a-Pa1 chimeric proteins were able to recognise AVR3a.

d) The Pa1-R3a chimeric protein confers resistance to the AVR3a^{KI} homozygous isolate of *P. infestans*, 7804.b. The DM-R3a chimeric protein does not provide resistance to 7804.b.

e) The Pa1-R3a chimera re-localises to PVCs in the presence of AVR3a^{KI}, as does the wild-type R3a protein. The unrecognised form of the effector, AVR3a^{EM}, does not cause this re-localisation. The DM-R3a chimeric protein remains cytoplasmic when expressed alone and in the presence of either form of AVR3a.

f) The Pa1-R3a may be stable when expressed *in planta*. Further western-blot experiments are needed to verify this result.

g) I have provided additional evidence that functional NB-LRRs develop through a process that requires concerted evolution of domains. Balancing selection

provides the diversity for the evolution of R3a but function is achieved only when domains are attuned to recognition and signalling.

CHAPTER 4

DETECTION OF THE VIRULENT FORM OF *PHYTOPHTHORA INFESTANS* EFFECTOR AVR3a FOLLOWING ARTIFICIAL EVOLUTION OF POTATO RESISTANCE GENE *R3a*

The study presented in this chapter resulted in a publication in PLOS One (Chapman and Stevens *et al*., 2014). The data presented in the publication were produced with the help of several colleagues from the James Hutton Institute and the University of Dundee. Work that has been carried out with others has been labelled clearly in the results and materials and methods sections. Sections of this chapter are direct reproductions from Chapman and Stevens *et al*. (2014).

4.1 INTRODUCTION

As described in **Chapters 1** and **3**, the LRR domain of plant resistance proteins has long been associated with effector recognition (Dinesh-Kumar *et al*., 2000, Axtell *et al*., 2001, Tornero *et al*., 2002). The results from the project detailed in **Chapter 3** suggest that the LRR domain of potato R3a is primarily involved in effector recognition, as a chimeric protein consisting of the CC-NB domain from an R3a paralog that does not recognise AVR3a^{KI} is able to recognise this effector form when combined with the wild-type R3a LRR domain. Moreover, this chimeric protein was able to provide resistance to a *P. infestans* isolate homozygous for AVR3a^{KI} to the same level as wild-type R3a and the recognition mechanism of this protein seems to mimic that of R3a.

The domain swapping study provided the rationale for targeting the LRR domain of R3a to enhance its recognition specificity. As wild-type R3a recognises only one of the two prevalent forms of the *P. infestans* effector AVR3a, it was thought artificial evolution of R3a could result in a gain-of-recognition of the normally virulent AVR3a^{EM} form. An evolved R protein which can recognise both forms of AVR3a could provide more durable resistance to *P. infestans* as there are a number of qualities of the effector which make it an ideal target for such resistance development.

AVR3a is an essential effector for *P. infestans* and it is present in every modern *P. infestans* isolate sequenced so far (Armstrong *et al*., 2005, Cárdenas *et al*., 2011). Stable silencing of AVR3a in the *P. infestans* isolate 88069 significantly reduces infection in susceptible *Solanum tuberosum* (potato) cv. Bintje and in the model Solanaceous plant species *Nicotiana benthamiana* (Bos *et al*., 2010; Vetukuri *et al*., 2011). There is limited sequence diversity of AVR3a in wild populations of *P. infestans*, with AVR3a^{KI} and AVR3a^{EM} forms being the most prevalent. However, two other forms of AVR3a have been identified in wild populations from the Toluca Valley in Mexico; AVR3aK^{80}I^{103}L^{139} (AVR3a^{KIL}) and AVR3aE^{80}M^{103}G^{124} (AVR3a^{EMG}) (Cárdenas *et al*., 2011; Seman *et al*., in preparation). These two forms appear to be rare and have only been found in combination with either AVR3a^{KI} or AVR3a^{EM}, thus far (Cárdenas *et al*., 2011 Seman *et al*., in preparation). Two paralogs of AVR3a have also been identified; Pex$^{147\text{-}2}$ and Pex$^{147\text{-}3}$, with only the latter being recognised by R3a (Armstrong *et al*., 2005). Moreover, a weak R3a-dependent response to AVR3a^{EM} can be observed under UV light (Bos *et al*., 2006), suggesting that this underlying, low level response could be enhanced to provide resistance.

DNA shuffling, also known as directed evolution, was first developed in the early 1990s and has since been used to generate a wide variety of novel genes and proteins (Stemmer, 1994). Artificial evolution has previously been used to alter the recognition specificity of the potato CC-NB-LRR resistance protein Rx to gain recognition of different strains of Potato virus X (PVX) and a distantly related virus, Poplar mosaic virus (PopMV) (Farnham and Baulcombe, 2006; Harris *et al*., 2013).

Two research groups have independently worked on artificially evolving R3a to extend its specificity to recognise both AVR3a forms. The authors of the first study introduced random mutations throughout the full length of R3a and recovered single amino acid changes in each of the three domains of R3a which increased hypersensitivity to $AVR3a^{EM}$ (Segretin *et al*., 2014). Whilst one of the R3a mutants also gained the ability to recognise the *P. capsici* effector protein PcAVR3a4, transient and stable expression of the R3a* mutants did not result in increased resistance to *P. infestans* in either *N. benthamiana* or potato plants (Segretin *et al*., 2014). Some amino acid mutations were found to cause low levels of auto-activity, particularly those mutations identified in the CC and NB domains of the R3a protein (Segretin *et al*., 2014).

The second study, the results of which are presented in this chapter, concentrated on PCR shuffling of only the LRR of R3a using an iterative process of error-prone PCR, DNA fragmentation, re-assembly and functional screening in *N. benthamiana* (Chapman and Stevens *et al*., 2014). Three R3a shuffled variants (R3a*) with varying degrees of gain-of-recognition of $AVR3a^{EM}$ were assessed in this study and the mechanisms underpinning the recognition were examined in detail (**Figure 4.1**). The R3a* variants were able to recognise $AVR3a^{EM}$ and the interaction between R3a* and $AVR3a^{EM}$ as well as $AVR3a^{KI}$ at the protein level recapitulated that of the wild-type R3a protein and

its cognate effector AVR3a^{KI} (Engelhardt *et al*., 2012; Chapman and Stevens *et al*., 2014).

My role in this project was to characterise three existing gain-of-recognition R3a* variants by comparing them mechanistically to wild-type R3a. **Figure 4.1** shows the iterative process of mutagenesis, shuffling and site-directed mutagenesis carried out and the different mutants generated from each round. The disease responses of each of the three R3a* shuffled variants under the control of the native R3a* promoter are also shown in **Figure 4.1**. The mutagenesis and shuffling of wild-type R3a was devised and carried out by Dr. Sean Chapman at The James Hutton Institute, prior to the beginning of my PhD, and is published as part of Chapman and Stevens *et al*. (2014).

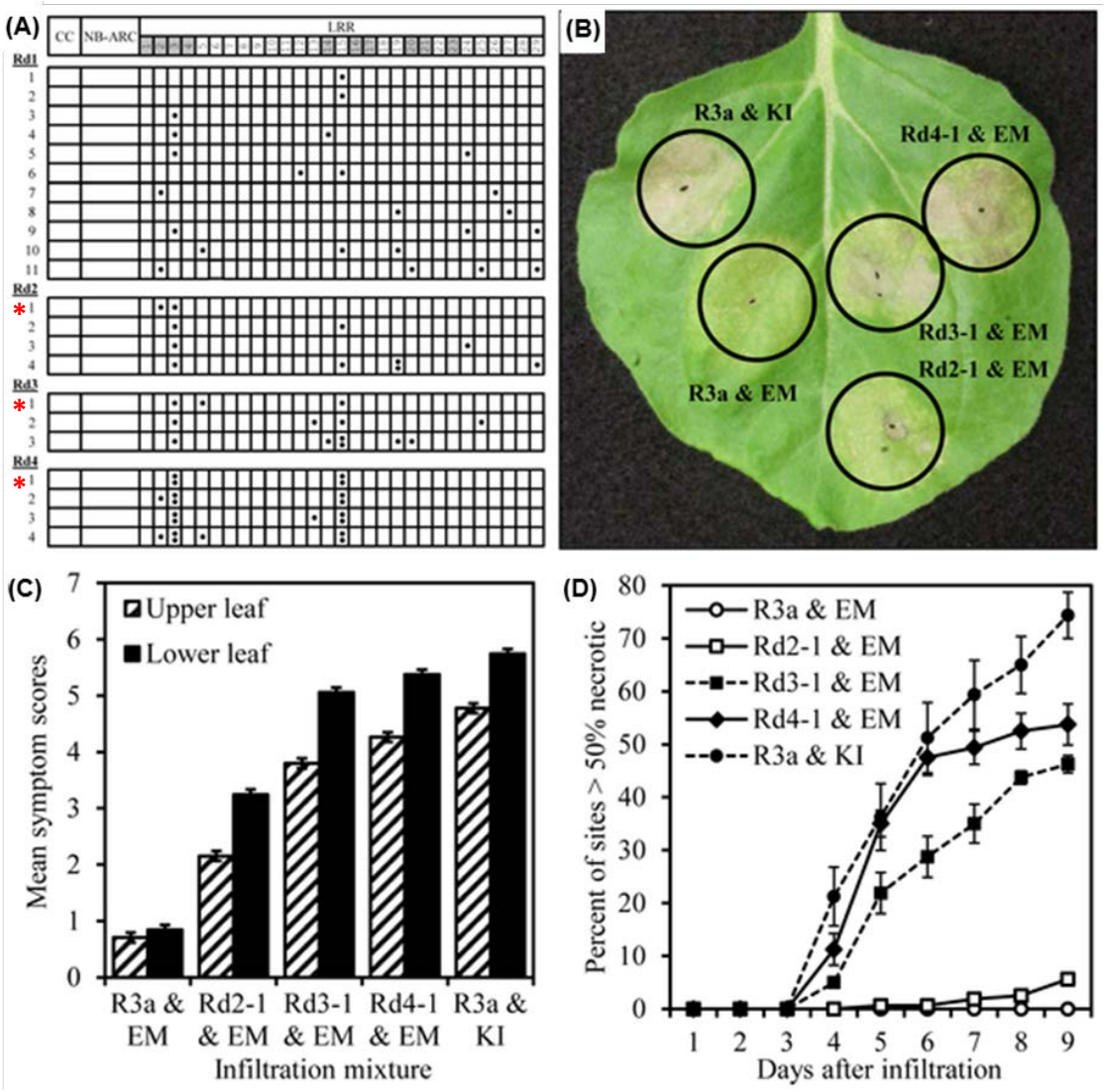

Figure 4.1: Four rounds of mutagenesis and shuffling identified R3a mutants with enhanced recognition of AVR3a^{EM} and disease responses (R3a*) (reproduced from Chapman and Stevens *et al*., 2014). (**A**) Schematic showing locations of non-synonymous mutations found in the LRRs of R3a* clones isolated from the four rounds of mutagenesis and shuffling (Rd1 to Rd4). LRRs containing amino acids under diversifying selection (Huang *et al*., 2005) are shaded above. The three R3a* clones referred to throughout this thesis are indicated by an *****. (**B**) Representative *N. benthamiana* leaf showing responses of best-performing clones from second, third and fourth rounds (Rd2-1, Rd3-1 & Rd4-1) to AVR3a^{EM} (EM), compared to responses of wild-type R3a to AVR3a^{KI} (KI) and AVR3a^{EM} five days after co-infiltration with resistance genes under the transcriptional control of the *R3a* promoter. (**C**) Mean disease scores from the four experiments, each of nine days duration, for different infiltration mixtures in upper (hatched) and lower (solid) paired leaves. Error bars show +/- standard error. (**D**) Time-course of percentage of sites showing necrosis development, greater than 50% necrosis of individual infiltrated sites, for the five infiltrated mixtures. Mean percentages of the four experiments. Each experiment includes data for 40 infiltration sites (upper and lower leaves combined) and error bars show +/- standard errors.

4.2 AIMS

The aims of the experiments detailed in this chapter were:

a) To investigate whether the R3a* shuffled variants Rd2-1, Rd3-1 and Rd4-1 are stable when expressed *in planta*.

b) To investigate the functional dependency of shuffled R3a* variants on the molecular chaperone molecules SGT1 and HSP90.

c) To investigate the cellular localisation of the products of shuffled R3a* variant genes in comparison to wild-type R3a when expressed alone and in the presence of recognised and unrecognised effector proteins as well as paralogs $PEX^{147\text{-}2}$ and $PEX^{147\text{-}3}$.

d) To investigate the cellular localisation of $AVR3a^{KI}$ and $AVR3a^{EM}$ when co-expressed with the products of shuffled R3a* variant genes.

e) To investigate the recognition specificity of products of R3a* shuffled variants when expressed with different AVR3a variants and paralogs $PEX^{147\text{-}2}$ and $PEX^{147\text{-}3}$.

f) To determine whether shuffled R3a* variants provide resistance to $AVR3a^{KI}$ homozygous and $AVR3a^{EM}$ homozygous strains of *Phytophthora infestans*.

4.3 RESULTS

4.3.1 R3a* shuffled variants are stable when expressed *in planta*

To show whether R3a* variants are stable *in planta*, western-blot analysis of protein extracts from inoculated *N. benthamiana* tissue was carried out. At least three independent biological replicates of the western-blot experiment showed that YFP-tagged Rd2-1, Rd3-1, Rd4-1 and wild-type R3a are stable when expressed *in planta* (**Figure 4.2**). As demonstrated for YFP-R3a wild-type fusions by Engelhardt *et al*. (2014), YFP-R3a* fusions did not elicit HRs alone or in the presence of $AVR3a^{KI}$ or $AVR3a^{EM}$, probably due to steric hindrance of the signalling domains of R3a (data not shown).

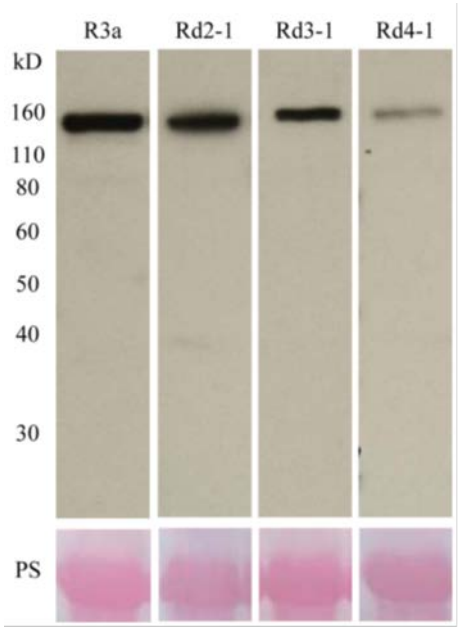

Figure 4.2: Stability of R3a* variants. Immunoblot probed with α-GFP following transient expression of wild-type YFP-R3a, YFP-Rd2-1, Rd3-1 and Rd4-1 in the absence of an effector in *N. benthamiana* at 2 d after inoculation. Protein sizes are indicated (in kilodaltons, kD), and protein loading is shown by Ponceau stain (PS). Western-blot carried out with the guidance of Dr. Stefan Engelhardt, image representative of three independent biological replicates.

4.3.2 R3a* recognition of AVR3a^{EM} is dependent on HSP90 and SGT1

Previous work by Bos *et al*. (2006) demonstrated that R3a-dependent recognition of AVR3a^{KI} involves both SGT1 (suppressor of the G2 allele of *skp1*) and HSP90 (heat shock protein 90) that are required for the activation of other R proteins (Liu *et al*., 2004; Azevedo *et al*., 2006). Their involvement in the AVR3a^{EM}-dependent responses was tested through Tobacco rattle virus (TRV)-based gene silencing of *SGT1*and *HSP90* in *N. benthamiana* with TRV-based expression of truncated GFP (eGFP) and mock-inoculated plants (Non-TRV) as controls. Three independent biological replicates for R3a, Rd2-1, Rd3-1 and one for Rd4-1, with infiltrations in two leaves of each of six plants per TRV-based silencing construct, revealed that both SGT1 and HSP90 are required to mediate a HR upon R3a*-based recognition of AVR3a^{KI} and AVR3a^{EM} (**Figure 4.3**). HRs were abolished for all infiltrations on TRV:*SGT1* inoculated plants and there were almost no HRs recorded on TRV:*HSP90* inoculated plants (**Figure 4.3**). The HRs were not affected on plants inoculated with TRV:*eGFP* and Non-TRV plants. Compared to TRV:*eGFP* inoculated plants, *SGT1* and *HSP90* silenced plants were morphologically stunted, a phenotype that has been reported previously (Bos *et al*., 2006). Nevertheless, upon infection with the bacterial pathogen *Erwinia amylovora* that produces an SGT1- and HSP90-independent non-host response in *N. benthamiana* (Gilroy *et al*., 2007), all plants were able to mount the expected cell death response (**Figure 4.3**). These data were highly reproducible and statistically highly significant. A one-way ANOVA showed that the recognition of AVR3a^{EM} by all R3a* variants and recognition of AVR3a^{KI} by all R3a* variants and wild-type R3a was reduced on TRV:*SGT1* inoculated plants ($F_{17,36}$ = 78.18, p < 0.001, n = 36). A second one-way ANOVA revealed that the recognition of AVR3a^{EM} by Rd2-1 and Rd3-1, and recognition of AVR3a^{KI} by Rd2-1, Rd3-1 and wild-type R3a was reduced on TRV:*HSP90* inoculated

plants ($F_{15,31}$ = 60.60, p < 0.001, n = 36). Rd4-1 was not included in this statistical analysis, as this variant was only included in one experiment with TRV:*HSP90* inoculated plants as the Rd4-1 variant was not available during earlier replicates of this experiment.

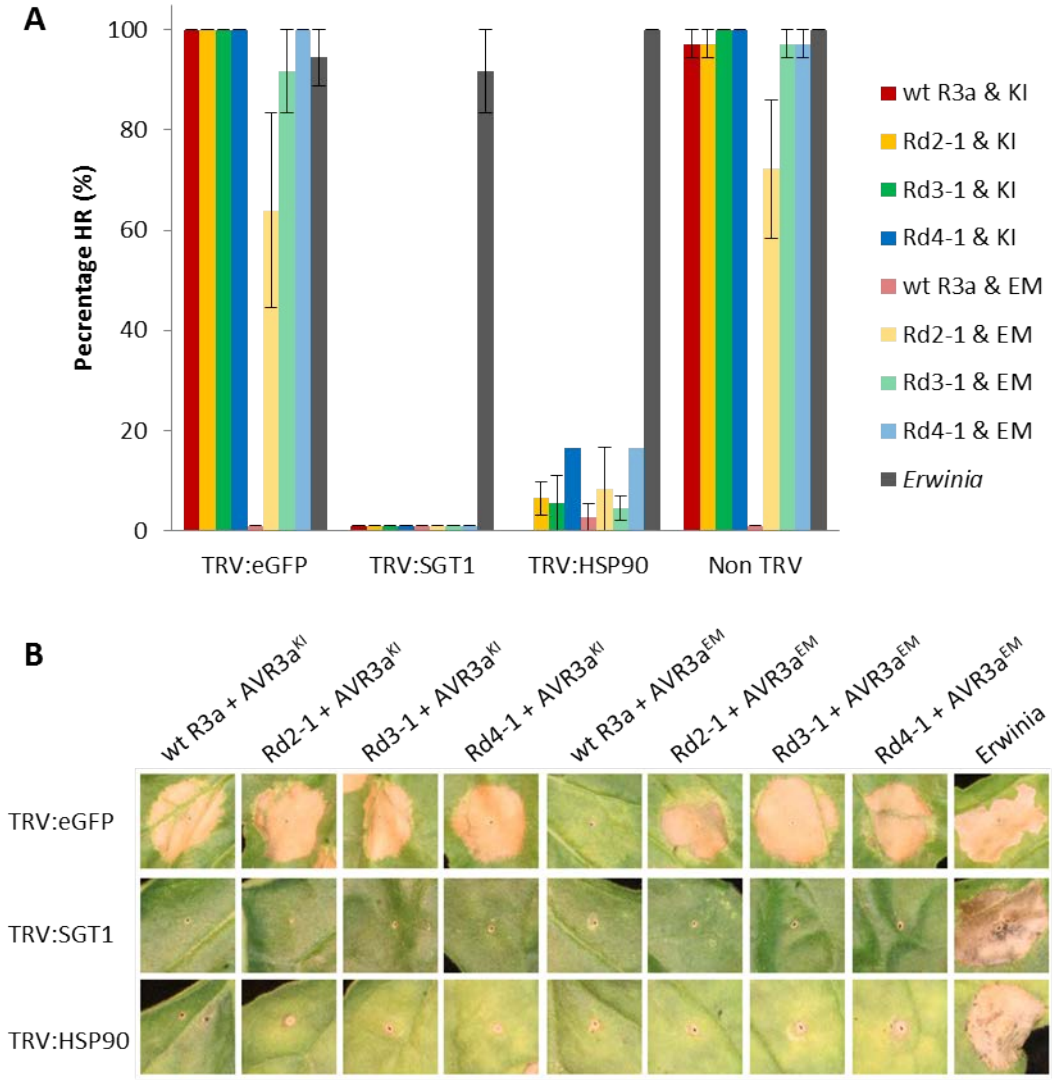

Figure 4.3: HR responses resulting from R3a* recognition of AVR3a^{EM} and AVR3a^{KI}, like those caused by wild-type R3a recognition of AVR3a^{KI}, are dependent on SGT1 and HSP90. (**A**) SGT1- and HSP90-silenced *N. benthamiana* plants were produced using TRV-based vectors. These plants and control plants inoculated with TRV:*eGFP* or mock-inoculated (Non TRV) were infiltrated with different combinations of *Agrobacterium* cultures designed to express R3a, R3a* variants, AVR3a^{KI} (KI) or AVR3a^{EM} (EM). The percentage of sites (n = 36) showing HR responses six days after infiltration was recorded. The graph shows the mean percentages from three independent biological replicates with the exception that the dependence on HSP90 of Rd4-1 responses was only tested in a single experiment. The non-host bacterial pathogen *Erwinia amylovora* was used as a control for an SGT1- and HSP90-independent HR response. Error bars show ± standard errors. Zero values have been transformed to 1% to

facilitate their observation. (**B**) Images show representative HR responses induced by each of the different mixtures on control TRV:eGFP inoculated plants, SGT1-silenced plants and HSP90-silenced plants. Images were taken 6 days after agroinfiltration under white light.

4.3.3 In a gain-of-mechanism, R3a* variants re-localise to late endosomes upon co-infiltration with AVR3a^{KI} or AVR3a^{EM}

In a previous study, Engelhardt *et al*. (2012) demonstrated that, upon recognition of AVR3a^{KI}, but not AVR3a^{EM}, wild-type R3a re-localises from the host cytoplasm to specific late endosomes that can be labelled with the cyan fluorescent protein marker PS1-CFP (Saint-Jean *et al*., 2010). This re-localisation was found to be a pre-requisite for subsequent HR development for untagged R3a co-expressed with AVR3a^{KI} (Engelhardt *et al*., 2012). To determine if R3a* variants with enhanced recognition of AVR3a^{EM} had gained the capacity to re-localise upon detection of AVR3a^{EM} and continued to exhibit this phenotype following detection of AVR3a^{KI}, I generated N-terminal fusions of R3a* variants Rd2-1, Rd3-1 and Rd4-1 with yellow fluorescent protein (YFP), with expression of these constructs driven by a 35S promoter. Following transient expression in *N. benthamiana*, all YFP-R3a/R3a* fusions when expressed by themselves displayed cytoplasmic localisations (**Figure 4.4**). I conducted the initial analysis on a Leica AOBS confocal microscope, whilst high-resolution images were taken with Dr. Petra Boevink on a Zeiss 710. In accordance with the observations described by Engelhardt *et al*. (2012), the localisation of YFP-R3a remained cytoplasmic upon co-infiltration with AVR3a^{EM}, but changed to fast-moving vesicles following recognition of AVR3a^{KI} (**Figure 4.4**). The YFP-R3a* fusions of Rd2-1 and Rd3-1 proteins maintained this mechanistically characteristic re-localisation following co-expression with AVR3a^{KI} (**Figure 4.4**). However, in contrast to YFP-R3a, all selected YFP-R3a*

variants also displayed highly reproducible re-localisation to PS1-CFP labelled vesicles after the perception of AVR3a^{EM} (**Figure 4.4** and **Figure 4.5**).

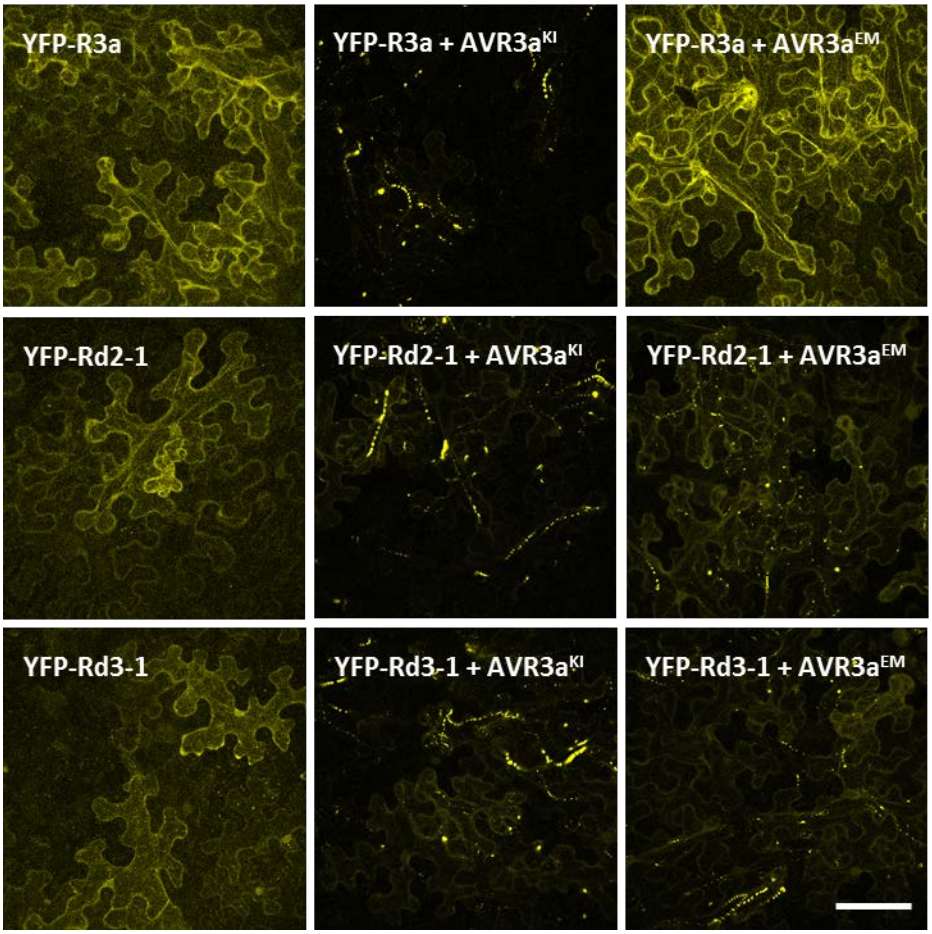

Figure 4.4: The cellular localisation of wild-type R3a and selected R3a* variants. YFP fusions to wild-type R3a and selected R3a* variants localise to the cytoplasm in the absence of AVR3a. YFP fusions to R3a* variants re-localise to vesicles after the perception of both AVR3a^{KI} and AVR3a^{EM}, whereas YFP-R3a remains cytoplasmic in the presence of AVR3a^{EM}. *N. benthamiana* leaves were infiltrated with cultures designed to express AVR3a^{KI}, AVR3a^{EM}, YFP fusions to R3a, Rd2-1 or Rd3-1. Leaf tissue was examined two days after infiltration under a confocal laser scanning microscope. Representative images are from five independent biological replicates. Scale bar = 50 µm.

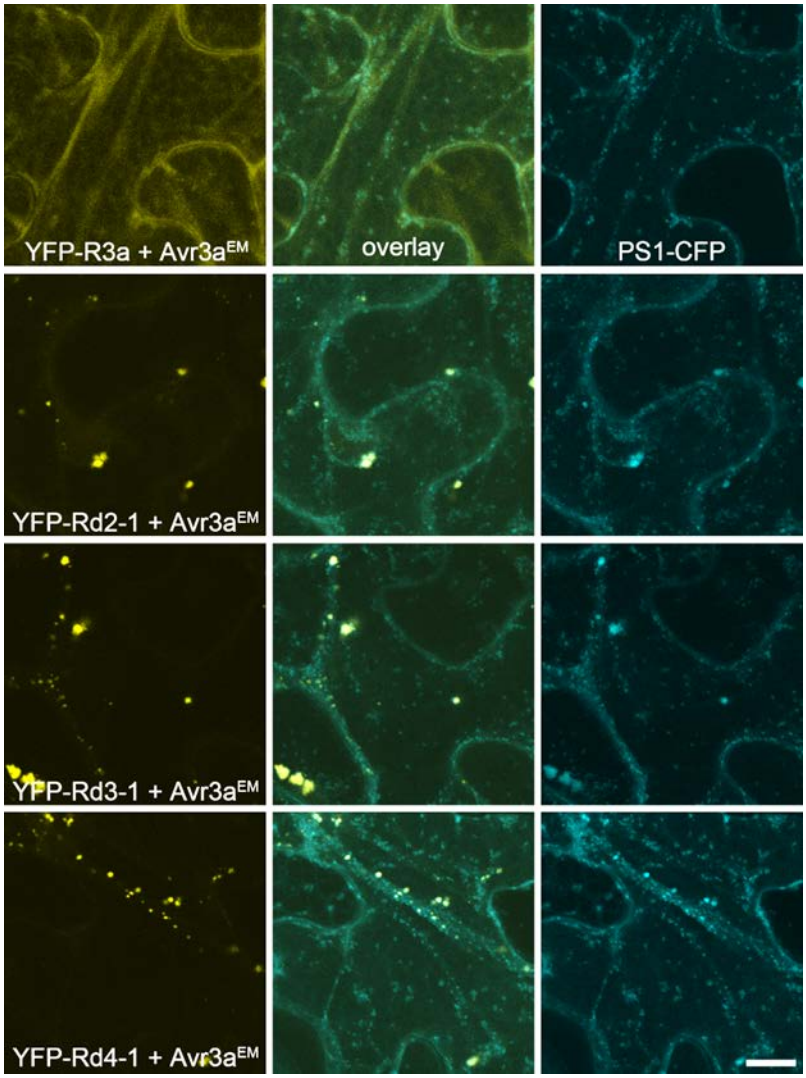

Figure 4.5: In the presence of AVR3a^{EM}, YFP fusions to R3a* variants, but not YFP-R3a, re-localise to vesicles labelled by the prevacuolar compartment marker PS1-CFP. *N. benthamiana* leaves were infiltrated with mixtures of cultures designed to express PS1-CFP, AVR3a^{EM} and YFP fusions to R3a, Rd2-1, Rd3-1 or Rd4-1. Leaf tissue was examined two days after infiltration under a confocal laser scanning microscope. The left-hand panel shows YFP signal, the right-hand panel CFP signal and the central panel displays the merged signals. Representative images are from three independent biological replicates. Scale bar = 10 µm. Images taken with the help of Dr. Petra Boevink.

4.3.4 AVR3a^{EM} re-localises to endosomes upon co-infiltration with R3a* variants but not with wild-type R3a

As shown by Engelhardt *et al*. (2012) AVR3a^{KI}, but not AVR3a^{EM}, also re-localises from the cytoplasm to endosomes upon co-expression with R3a. This was demonstrated by N-terminal fusions of AVR3a^{KI} and AVR3a^{EM} to green fluorescent protein as well as by bi-molecular fluorescence complementation (BiFC, or split-YFP) assays (Walter *et al*., 2004; Bos *et al*., 2010; Engelhardt *et al*., 2012). The latter revealed that wild-type R3a and AVR3a^{KI} are found in close proximity at PS1-CFP labelled vesicles (Engelhardt *et al*., 2012). To investigate if the vesicular co-association of R3a and AVR3a^{KI} was extended to the R3a* variants, BiFC was used to analyse and localise protein–protein interactions *in planta*. As described previously for wild-type R3a (Engelhardt *et al*., 2012), the N-terminal portion of YFP, YN, was fused to the N-terminal end of the R3a* variants Rd2-1, Rd3-1 and Rd4-1. The constructs used to express the C-terminal portion of YFP, YC, fused to AVR3a^{KI} and AVR3a^{EM}, were as described previously (Engelhardt *et al*., 2012) with all constructs being transiently expressed in *N. benthamiana* from the 35S promoter. In accordance with previous findings (Engelhardt *et al*., 2012), co-expression of YN-R3a with YC-AVR3a^{KI} generated strong YFP fluorescence, whereas co-expression with YC-AVR3a^{EM} generated no detectable YFP fluorescence (**Figure 4.6**). Like the YN fusion to wild-type R3a, all the YN-R3a* fusions when co-expressed with AVR3a^{KI} gave strong, punctate, YFP signals (**Figure 4.6**). However, unlike the wild-type R3a fusion, R3a* fusions also gave YFP fluorescence signals at PS1-CFP labelled vesicles when co-expressed with AVR3a^{EM} (**Figure 4.6** and **Figure 4.7**). This indicates that AVR3a^{EM} is also within close proximity of the re-localised R3a* gene products. Thus, in line with the gain of recognition of AVR3a^{EM} by the R3a* variants and subsequent

necrosis responses, the R3a* variants and AVR3a^{EM} show the same mechanistic re-localisation as observed for R3a and AVR3a^{KI}. Experimental design and the set-up of experiments were performed by myself, whilst the imaging was performed with Dr. Petra Boevink.

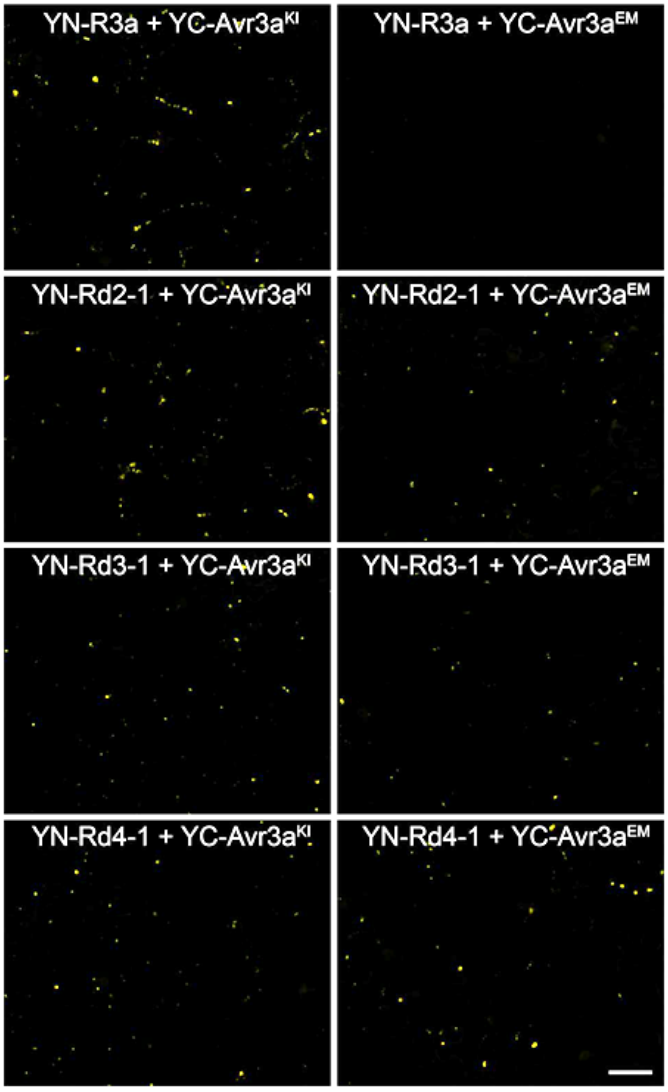

Figure 4.6: Both YC-AVR3a^{KI} and YC-AVR3a^{EM} when co-expressed with YN-R3a* fusions yield vesicle-associated YFP fluorescence similar to YC-AVR3a^{KI} and YN-R3a, whereas YC-AVR3a^{EM} and YN-R3a do not. Two days after infiltration of mixtures of *Agrobacterium* cultures designed to express YC-AVR3a^{KI}, YC-AVR3^{EM}, YN-R3a or YN fusions to the R3a* variants, infiltrated *N. benthamiana* leaf tissue was examined under a confocal laser scanning microscope. Representative images from two biological replicates. Scale bar = 50 µm. Images taken with the help of Dr. Petra Boevink.

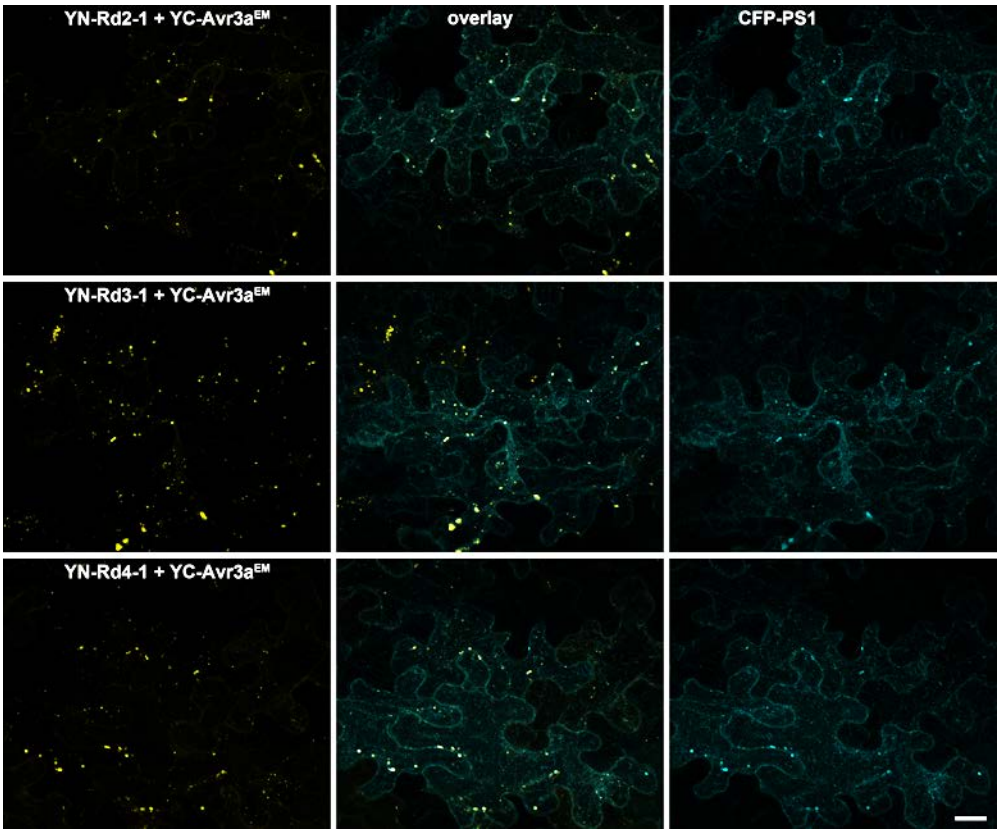

Figure 4.7: YC-AVR3a^{EM} reconstitutes YFP fluorescence with YN fusions to the R3a* variants at vesicles labelled by the prevacuolar compartment marker PS1-CFP. Generation of the YFP signal indicates that AVR3a^{EM} and the R3a* variants are in close proximity at the vesicles. *N. benthamiana* leaves were infiltrated with mixtures of cultures designed to express PS1-CFP, YC-AVR3a^{EM} and YN fusions to Rd2-1, Rd3-1 or Rd4-1. Leaf tissue was examined 2 d after infiltration under a confocal laser scanning microscope. Left-hand panel, YFP signal; right-hand panel, CFP signal; central panel, merged signals. Representative images from three independent biological replicates. Scale bar = 20 µm. Images taken with the help of Dr. Petra Boevink.

4.3.5 The recognition specificity of products of R3a* shuffled variants when expressed with different AVR3a variants and paralogs.

As mentioned previously, naturally occurring AVR3a variants AVR3a^{KIL} and AVR3a^{EMG} have been identified in *P. infestans* isolates collected from South America (Cárdenas *et al*., 2011; Seman *et al*., in preparation). Together with the paralogous sequences PEX$^{147\text{-}2}$ and PEX$^{147\text{-}3}$ characterised by Armstrong *et al*. (2005), there is limited sequence variation evident for the *Avr3a* gene family. To ascertain if these variations in the effector sequence are potentially in response to naturally occurring resistance genes that recognise, for example, both AVR3a^{KI} and AVR3a^{EM}, I studied their recognition patterns with wild type R3a and R3a* variants in three independent, transient co-infiltration assays in *N. benthamiana*. The recognition of AVR3a^{KI}, AVR3a^{KIL} and Pex$^{147\text{-}3}$ by R3a* variants Rd2-1 and Rd3-1 is similar to that of wild-type R3a, in that all of the tested R proteins recognised these AVR3a variants, with no statistically significant differences when tested in one-way ANOVA (AVR3a^{KI}: $F_{2,6} = 0.11$, n = 36, $p = 0.900$; AVR3a^{KIL}: $F_{2,6} = 0.35$, n = 36, $p = 0.719$; Pex$^{147\text{-}3}$: $F_{2,6} = 0.06$, n = 36, $p = 0.945$; **Figure 4.8**). However, highly statistically significant differences were observed in the recognition of AVR3a^{EM} and AVR3a^{EMG} by Rd2-1 and Rd3-1 when compared to wild-type R3a (AVR3a^{EM}: $F_{2,6} = 181.12$, n = 36, $p < 0.001$; AVR3a^{EMG}: $F_{2,6} = 28.53$, n = 36, $p = 0.001$; **Figure 4.8**). Both of the R3a* variants tested recognised AVR3a^{EM} and AVR3a^{EMG}, whilst wild-type R3a did not (**Figure 4.8**). No HRs were recorded for co-infiltrations with Pex$^{147\text{-}2}$ and wild-type R3a, Rd2-1 or Rd3-1 and in the absence of an effector (**Figure 4.8**).

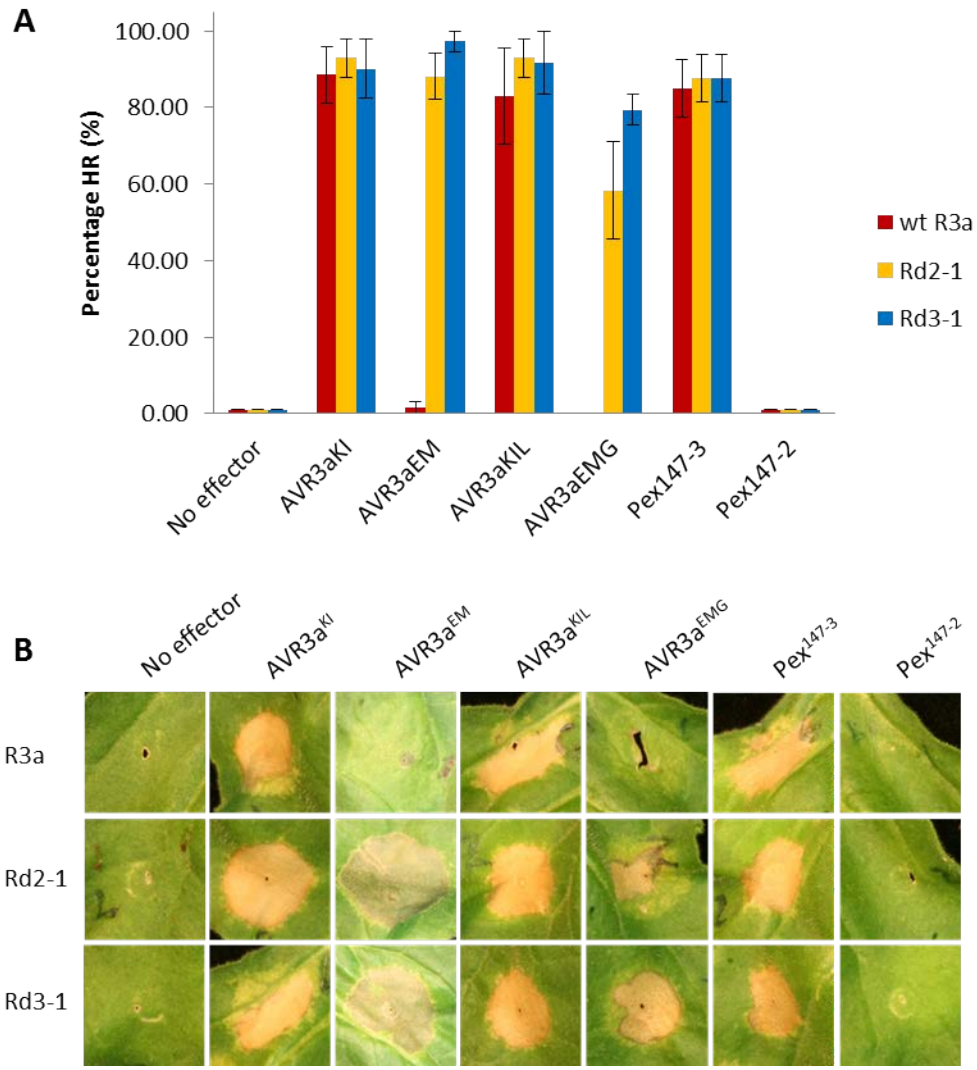

Figure 4.8: Recognition spectra of wild-type R3a and R3a* variants when expressed with AVR3a variants and paralogs. (**A**) Mean percentage HRs of wild-type R3a, Rd2-1 and Rd3-1 in the presence of various AVR3a variants and paralogs. Indicated constructs were transiently co-expressed by agro-infiltration in *N. benthamiana* and HRs were measured 3 days after agro-infiltration from three independent biological replicates. Error bars indicate ± SEM. Zero values have been transformed to 1% to facilitate their observation. (**B**) Images show representative HR responses induced by indicated constructs upon transient co-expression by agro-infiltration in *N. benthamiana*. Images were taken 3 days after agro-infiltration under white light.

4.3.6 The cellular localisation of products of R3a* shuffled variants when expressed with different AVR3a variants and paralogs.

To study if the recognition of AVR3a variants and paralogs by selected R3a* variants was accompanied by re-localisation to vesicles, the cellular localisation of YFP-fusions to Rd2-1 and Rd3-1 was studied with confocal microscopy. The N-terminal fusions of wild-type R3a and R3a* variants Rd2-1 and Rd3-1 with yellow fluorescent protein (YFP) described previously, were transiently expressed in *N. benthamiana*, along with $AVR3a^{KIL}$, $AVR3a^{EMG}$, Pex^{147-3}, or Pex^{147-2}. All YFP-R3a/R3a* fusions displayed cytoplasmic localisations when expressed by themselves (**Figure 4.9**). In accordance with the observations described by Engelhardt *et al*. (2012), co-expression of the YFP-fusion of wild-type R3a with recognised effector forms, in this case $AVR3a^{KIL}$ and Pex^{147-3}, results in a rapid re-localisation to fast-moving vesicles (**Figure 4.9**). However, in the presence of $AVR3a^{EMG}$, YFP-R3a remained cytoplasmic (**Figure 4.9**). In contrast, YFP-fusions of Rd2-1 and Rd3-1 re-localised to vesicles in the presence of $AVR3a^{EMG}$ (**Figure 4.9**). YFP-R3a* variants retained this characteristic re-localisation in the presence of $AVR3a^{KIL}$ and Pex^{147-3}, but not in the presence of Pex^{147-2} (**Figure 4.9**).

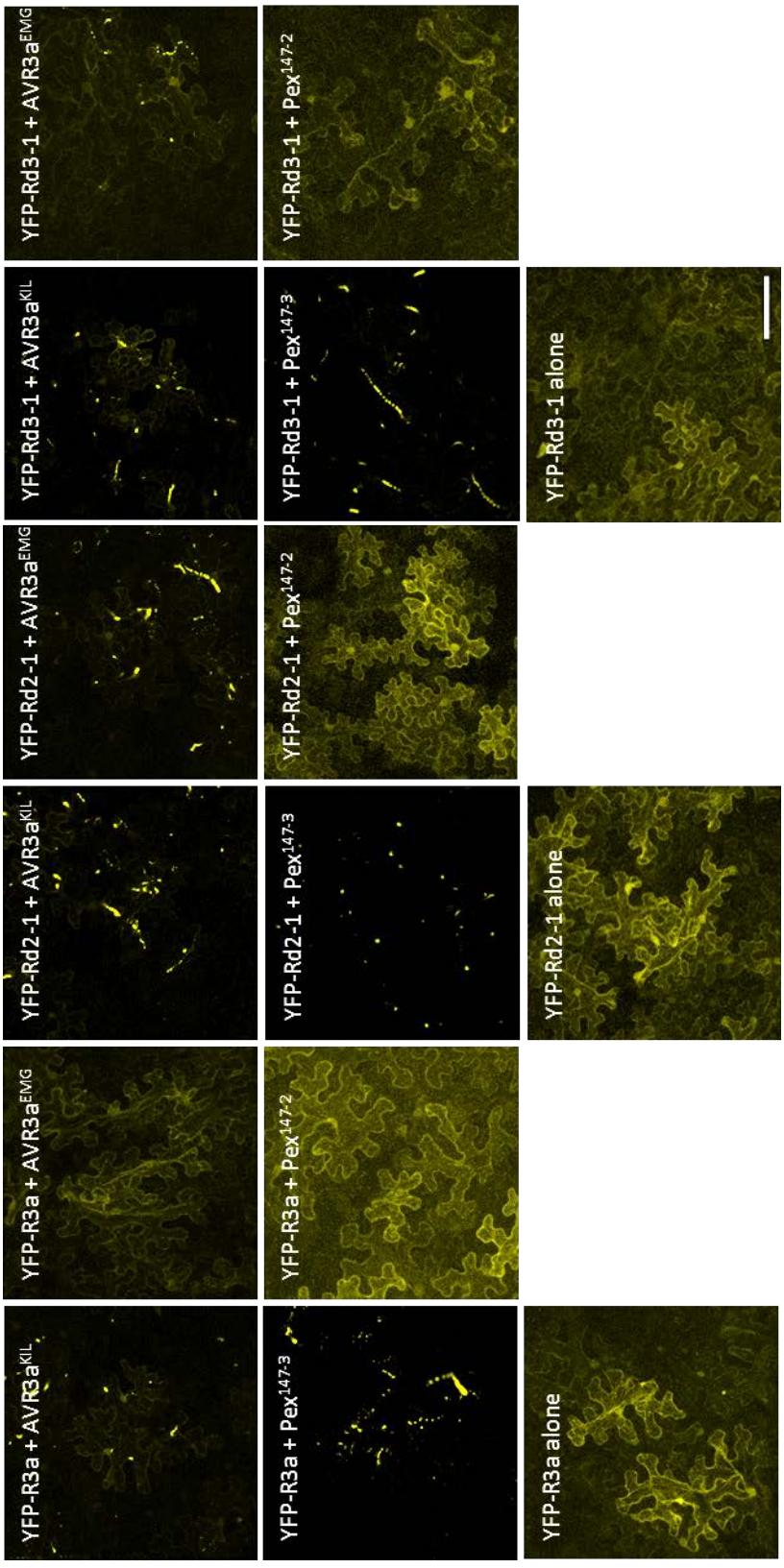

Figure 4.9: Cellular localisation of wt R3a and R3a* variants in the presence of different AVR3a variants and paralogs. YFP-fused wt R3a and R3a* variants Rd2-1 and Rd3-1 re-localise to vesicles in the presence of AVR3a^{KIL} and Pex$^{147-3}$, but remain cytoplasmic in the presence of Pex$^{147-2}$ and in the absence of an effector. YFP-fused Rd2-1 and Rd3-1, but not YFP-R3a, re-localise to vesicles in the presence of AVR3a^{EMG}. Two days after infiltration of mixtures of *Agrobacterium tumefaciens* cultures expressing AVR3a^{KI}, AVR3a^{EM}, YFP-R3a, YFP-Pa1-R3a or

YFP-DM-Pa1 fusions, *N. benthamiana* leaves were examined under a confocal laser scanning microscope. Representative images are from three independent biological replicates. Scale bar = 50 µm.

4.3.7 R3a* variants maintain resistance towards AVR3a^{KI}-expressing *P. infestans* isolates but have not gained resistance towards AVR3a^{EM} homozygous isolates

To evaluate if R3a* variants with gain of AVR3a^{EM} recognition and re-localisation mechanism yield effective disease resistance, transient and stable expression systems were utilised. *Agrobacterium tumefaciens* transient assays (ATTAs) in *N. benthamiana* have successfully been used to demonstrate function for late blight resistance gene products such as R2, Rpi_STO1 (Saunders *et al*., 2012) and R3b (Li *et al*., 2011). Selected R3a* clones Rd2-1, Rd3-1 and Rd4-1 were transiently expressed in *N. benthamiana* using the native R3a promoter in ATTAs alongside wild-type R3a and an empty vector control. Infiltrated leaf areas were challenged two days after infiltration with AVR3a^{KI} or AVR3a^{EM} homozygous *P. infestans* isolates via drop inoculation. Disease progression was monitored by measuring visible lesion diameters in multiple independent experiments. In three independent experiments ATTA sites were inoculated with the AVR3a^{KI} homozygous *P. infestans* isolate 7804.b. A one-way ANOVA performed on the data from the three independent biological replicates showed that the wild-type R3a and the R3a* variants led to highly significantly reduced leaf colonisation of *P. infestans* relative to the empty vector control and there were no significant differences between the different R3a forms ($F_{3,8}$ = 7.84, p = 0.009, n = 80, **Figure 4.10**). This result indicates that the selected mutations in the LRR do not impair the resistance induced by AVR3a^{KI}.

Similar ATTA sites were inoculated with the AVR3a^{EM} homozygous isolate 88069 (Bos *et al*., 2010) in four independent experiments. A one-way ANOVA performed on the data from the four independent biological replicates showed that there were no significant differences in *P. infestans* spread between any of the R3a forms and the empty vector control ($F_{4,15}$ = 1.00, p = 0.438, n = 104, **Figure 4.10**). Co-infiltrations of R3a, R3a*, AVR3a^{KI} and AVR3a^{EM} constructs were carried out contemporaneously in all experiments to confirm that the conditions were conducive to HR development (data not shown).

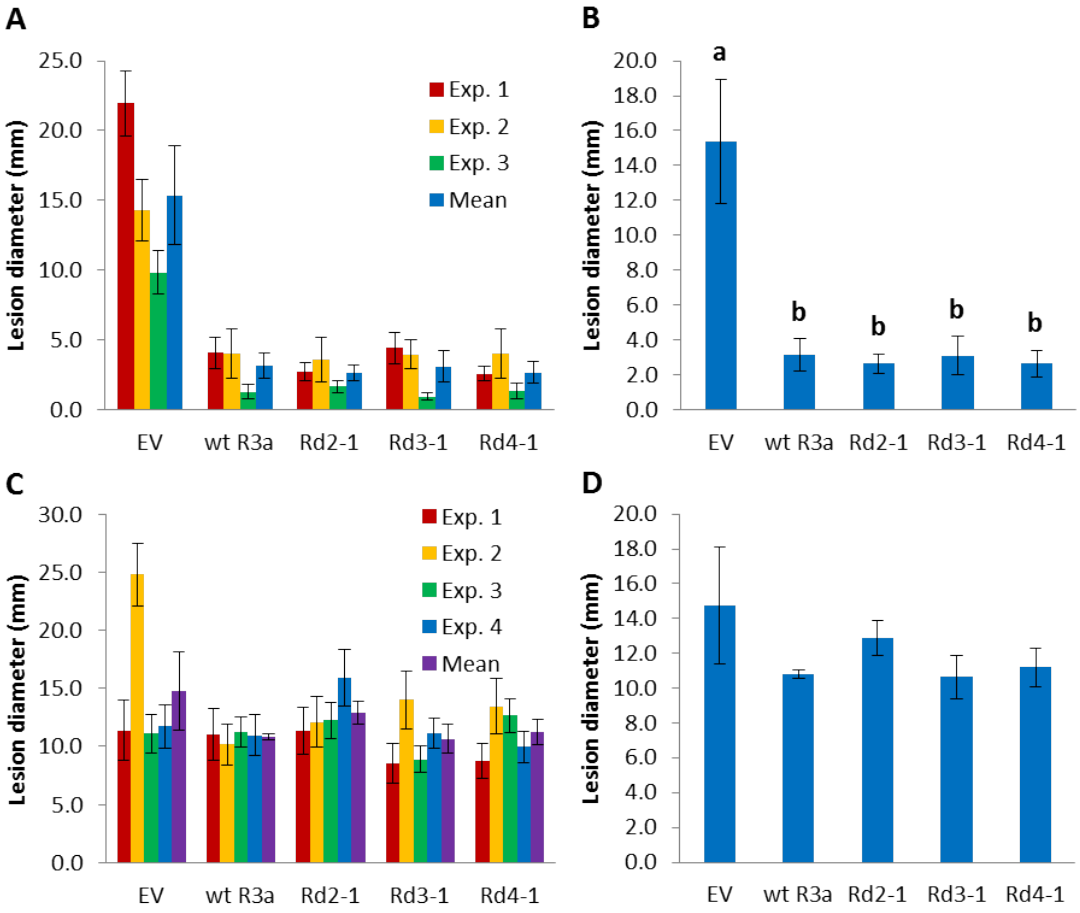

Figure 4.10: R3a* variants maintain resistance towards AVR3a^{KI}-expressing *P. infestans* isolates but have not gained resistance towards AVR3a^{EM} homozygous isolates. (**A**) Lesion diameters measured 7-12 days after drop inoculation of agro-infiltrated areas with *P. infestans* strain 7804.b (KI/KI): the mean lesion diameter from each of three independent experiments is shown alongside the mean from the three independent biological replicates. (**B**) Mean lesion diameters measured 7-12 days after drop inoculation of agro-infiltrated areas with *P. infestans* strain 7804.b (KI/KI) from three independent biological replicates. Shared letters above the bars indicate that those means are not significantly different, grouping information generated with the Tukey Method (95 %), n = 80, $F_{4,10}$ = 10.11, p = 0.002. (**C**) Lesion diameters measured 7-12 days after drop inoculation of agro-infiltrated areas with *P. infestans* strain 88069 (EM/EM): the mean lesion diameter from each of four independent experiments is shown alongside the mean from the four independent biological replicates. (**D**) Mean lesion diameters measured 7-12 days after drop inoculation of agro-infiltrated areas with *P. infestans* strain 88069 (EM/EM) from four independent biological replicates: n = 104, $F_{4,15}$ = 1.00, *p* = 0.438. All error bars indicate ± SE_M, EV = empty vector control. The mean lesion diameter shown in (**A**) and (**B**) is the average of the three mean values from the three experimental replicates shown in (**A**). The mean lesion diameter shown in (**C**) and (**D**) is the average of the four mean values from the four experimental replicates shown in (**C**).

4.4 DISCUSSION

Phytophthora infestans has the ability to rapidly overcome novel resistance genes deployed in the field due to its "high evolutionary potential" (Raffaelle *et al*., 2010). There are more than 560 RXLR-type effector genes annotated in the *P. infestans* genome, and their location, often in gene-poor and repeat-rich regions, is thought to drive their evolution and diversity (Haas *et al*., 2009). There are a number of ways in which an oomycete RXLR-type effector can avoid detection by an *R* gene product; by transcriptional silencing (Gilroy *et al*., 2011a; Rietman *et al*., 2012), mutations which cause a frameshift and thus a truncated effector protein that evades R protein recognition (van Poppel *et al*., 2008), by utilising suppressers of immune responses that perturb specific ETI responses (Wang *et al*., 2011) and/or sequence diversity (Armstrong *et al*., 2005; Gilroy *et al*., 2011a).

The *P. infestans* AVR3a effector exists in two forms, with the AVR3a^{KI} allele determining avirulence on plants carrying *R3a* whereas AVR3a^{EM} evades recognition and promotes virulence. It is thought that only AVR3a^{KI} was present in the *P. infestans* strain responsible for the outbreak of late blight disease leading to the Irish Potato Famine in the 1840s (Yoshida *et al*., 2013). It is conceivable that the AVR3a^{EM} allele came to dominate in *P. infestans* populations once the resistance gene *R3a* was more widely deployed in potato cultivars such as Pentland Dell, as the resistances it harboured were defeated within four years of its release (White and Shaw, 2010).

Breeding natural resistance genes into potato has traditionally been a laborious process, illustrated by the relatively narrow range of genetic diversity within current clonal potato varieties. It is hoped that by mining the vast amounts of information

being generated from studies on R protein-effector interactions, novel resistance proteins with enhanced recognition specificities can be rationally designed and deployed in transgenic plants. Functional wild-type R3a, a homolog of the tomato *I2* resistance gene that controls resistance to races of the fungus *Fusarium oxysporum* (Ori *et al*., 1997), was cloned alongside three paralogous sequences that provided insight into amino acid positions under diversification (Huang *et al*., 2005). Thirteen amino acid residues of R3a were found to be under significant diversifying selection, with one of these residues being situated in the CC domain, whilst the rest were within the LRR domain (Huang *et al*., 2005). The residues under diversifying selection in the LRR domain cluster around two regions spanning LRRs #1 to #4 and #14 to #23 (Huang *et al*., 2005). Moreover, the results from the project detailed in **Chapter 3** suggested that the LRR domain of R3a is involved in effector recognition and resistance to recognized effectors.

The approach taken in this study (Chapman and Stevens *et al*., 2014) was to artificially evolve the LRR of *R3a* using a combination of random mutagenesis, DNA shuffling and targeted mutagenesis to enhance AVR3a^{EM} recognition by R3a* variants. DNA shuffling, which mimics the natural evolutionary processes of mutation, recombination and selection at an accelerated pace, has proven a highly effective method for evolving new specificities/properties for a wide range of proteins that cannot be rationally designed and is of particular use in identifying mutations that are beneficial in combination (Stemmer, 1994).

In the primary screen for the R3a* variants detailed in this chapter, eleven mutants with enhanced AVR3a^{EM} recognition were identified, which had 23 different amino acid substitutions in total. However, only three of these (R618Q, K920E, Q931R), found

in the clones with single amino acid substitutions, are known to contribute to the improved phenotype (Chapman and Stevens *et al*., 2014). The complementary study performed by Segretin *et al*. (2014) identified six amino acid substitutions in the LRR domain that enhanced AVR3a^{EM} recognition: two of these (L668P, K920E) were also found in the primary screen by Chapman and Stevens *et al*. (2014). As different mutations in the LRR of R3a were identified by both Segretin *et al*. (2014) and Chapman and Stevens *et al*. (2014), it can be inferred that neither screen was exhaustive. Mutations leading to amino acid substitutions in LRRs #3 and #15 were prevalent in gain of recognition of AVR3a^{EM} mutants identified from the first round of screening, reinforcing the idea that these residues are important for effector recognition as these were also found to be under diversifying selection by Huang *et al*. (2005).

In the study published in Chapman and Stevens *et al*. (2014), we used site-directed mutagenesis in order to bring together combinatorially beneficial mutations, identified during the random mutagenesis stage, within LRRs that are in close proximity. Screening of site-directed mutants resulted in the identification of variants with enhanced recognition of AVR3a^{EM} when compared to second-round shuffling variants (Chapman and Stevens *et al*., 2014). The site-directed R3a* mutant selected for further analysis contained two amino acid changes in close proximity in each of LRRs #3 and #15; a combination that would have been difficult to obtain through DNA shuffling. As shown by Segretin *et al*. (2014) for the R3a mutants with enhanced AVR3a^{EM} recognition, there was no reduction in the AVR3a^{KI} recognition responses of the R3a* variants (Chapman and Stevens *et al*., 2014).

The recognition of AVR3a^{EM} by the three R3a* variants recapitulates the mechanistic processes of recognition of AVR3a^{KI} by the wild-type R3a protein. It has previously been reported (Bos *et al*., 2006) that the HR triggered by R3a-mediated recognition of AVR3a^{KI} is dependent on the ubiquitin ligase associated protein SGT1 and HSP90. VIGS of SGT1 and, to a lesser degree, of HSP90, inhibited the cell death responses induced by recognition of AVR3a^{EM} by R3a* variants (**Figure 4.3**). Similarly, it has been shown that wild-type R3a re-localises from the cytoplasm to late endosomal compartments when co-expressed with AVR3a^{KI}, but not when co-expressed with AVR3a^{EM} (Engelhardt *et al*., 2012). The three R3a* variants tested in this study still re-localised to endosomal compartments when co-expressed with AVR3a^{KI} and, importantly, also re-localised to the same vesicles when co-expressed with AVR3a^{EM} and AVR3a^{EMG} (**Figure 4.4, Figure 4.5** and **Figure 4.9**).

Engelhardt *et al*. (2012) also showed that the effector AVR3a^{KI} itself re-localises from the cytoplasm to endosomes when co-expressed with wild-type R3a and is in close physical proximity to R3a, whereas AVR3a^{EM} remains localised in the cytoplasm. The BiFC experiments show that AVR3a^{KI} and the normally unrecognized form AVR3a^{EM} both traffic from the cytoplasm to vesicles when co-expressed with the R3a* variants (**Figure 4.6** and **Figure 4.7**). This re-localisation of R3a and AVR3^{KI} was shown to be a prerequisite for the development of the HR (Engelhardt *et al*., 2012). These results show that the recognition of AVR3a^{EM} by the three R3a* variants is mechanistically similar to the wild-type R3a-mediated recognition of AVR3a^{KI}. Although a direct interaction between the R3a and AVR3a^{KI} proteins has not been shown (Engelhardt *et al*., 2012), our results indicate that R3a* variants interact with AVR3a^{EM} as part of the

same protein complex where wild-type R3a and AVR3a converge. This could be through interaction with an as yet unidentified protein guardee of R3a.

Although the R3a* variants characterised in this study responded to AVR3a^{EM} and yielded HR responses when the effector was transiently expressed via *Agrobacterium* in *N. benthamiana*, critically they only provided resistance to *P. infestans* isolates that express AVR3a^{KI} and not to an isolate that expresses only AVR3a^{EM} (**Figure 4.10**). The results from ATTAs showing a lack of R3a*-mediated resistance to the AVR3a^{EM} homozygous isolate 88069 were mirrored by experiments in which stable transgenic potato plants expressing the R3a* genes were challenged with two different Mexican *P. infestans* isolates (Chapman and Stevens *et al*., 2014). Stable R3a* transgenic plants demonstrated high levels of resistance towards isolate P6752a, which is heterozygous for AVR3a^{KI} and AVR3a^{EM} (Chapman and Stevens *et al*., 2014). However, the same transgenic lines were unable to control disease development of the US-8 BF-6 isolate, an AVR3a^{EM} homozygote (Chapman and Stevens *et al*., 2014). Together, these results indicate that the mutations within the LRR domains of R3a* variants do not negatively impact on resistance towards *P. infestans* isolates expressing AVR3a^{KI}, but do not confer resistance to *P. infestans* isolates homozygous for AVR3a^{EM}. Similarly, the R3a mutants generated by Segretin *et al*. (2014) failed to confer resistance to AVR3a^{EM}-expressing isolates of *P. infestans*. The fact that the results of blight testing on stably transformed potato plants corroborate those results produced during ATTA experiments in *N. benthamiana* shows that ATTA experiments can be excellent proxy systems for disease testing (Vleeshouwers *et al*., 1999). During an ATTA experiment, PTI is unavoidably elicited due to co-infiltration of *Agrobacterium* cultures, whilst wounding occurs when leaves are detached from *N. benthamiana* plants. Both of these

events activate signalling cascades which could result in cross-talk with other plant defence responses (León *et al*., 2001) and could potentially interfere with the ETI signalling mediated by R protein activation. However, the results from our ATTA experiments were similar to those where *Agrobacterium*-triggered PTI and plant wounding were not factors in the experimental process. Furthermore, these results indicate that either the virulence target of AVR3a is conserved between *N. benthamiana* and potato, or the protein interaction between R3a and AVR3a is direct, which, as discussed previously, has never been shown. However, it appears that signalling of wild-type R3a and the R3a* variants functions in *N. benthamiana* as well as it does in potato.

It has previously been suggested that the strength of the HR in response to *P. infestans* in host species correlates with resistance levels to the pathogen (Vleeshouwers *et al*., 2000). In *P. infestans* non-host species (*Arabidopsis*, *Nicotiana tabacum*, *Raphanus sativa* and *Mirabilis jalapa*) and in fully resistant *Solanum* species, the HR is rapid and robust (Vleeshouwers *et al*., 2000). However, the HR was significantly delayed in partially resistant *Solanum* species, indicating that the strength of the HR can correlate with resistance to *P. infestans* (Vleeshouwers *et al*., 2000). Conversely, there is evidence for the uncoupling of the HR response and disease resistance from other plant/pathogen systems (Bendahmane *et al*., 1999; Piffanelli *et al*., 2002; Király and Király, 2006). Yu *et al*. (1998) demonstrated that mutations within the DND (Defence, No Death) locus of *Arabidopsis* result in induction of resistance responses to avirulent *Pseudomonas syringae,* without the induction of cell death. In a study by Bendahmane *et al*. (1999), potato *Rx* was found to confer extreme resistance to PVX when expressed as a transgene in potato or *Nicotiana* species and this resistance was not associated

with cell death. The same authors reported that Rx is able to mediate cell death upon expression of the viral elicitor from a vector rather than from the PVX genome (Bendahmane *et al*., 1999).

The recent work on the artificial evolution of potato Rx has highlighted the need for a stepwise approach to fine-tune the manner in which different domains of an R protein co-operate with each other (Harris *et al*., 2013). A previous study identified four single amino acid LRR mutants of Rx, which conferred broader disease resistance specificities than the wild-type protein (Farnham and Baulcombe, 2006). These mutants retained the ability to recognise an avirulent strain of PVX but also gained resistance to a PVX strain which is virulent on plants harbouring the original *Rx* (Farnham and Baulcombe, 2006). In addition, the Rx mutants were found to be resistant to the distantly related poplar mosaic virus (PopMV) (Farnham and Baulcombe, 2006). However, one of the mutants exhibited a trailing necrosis which resulted in the death of the plant upon inoculation with PopMV, indicating a cost to the broad-spectrum disease resistance mediated by the mutations within the LRR domain (Harris *et al*., 2013). A second mutational analysis targeted the CC-NB-ARC1-ARC2 domain of Rx (whilst maintaining the mutations introduced in the LRR domain) and resulted in the identification of four Rx mutants with enhanced responses to PopMV. In three of these mutants, the improved broad-spectrum resistance was found to be due to single amino acid changes, whilst the fourth mutant possessed two amino acid substitutions (Harris *et al*., 2013). All of the mutations were located in the vicinity of the nucleotide binding pocket of Rx (Harris *et al*., 2013).

The two independent studies on the directed evolution of *R3a* took different approaches, whilst the study by Chapman and Stevens *et al*. (2014) focussed on

mutating only the LRR domain. Segretin *et al*. (2014) introduced mutations throughout the entire length of R3a. In the latter study, out of eight single amino acid substitutions which resulted in enhanced response to AVR3a^{EM}, six were located in the LRR domain, one occurred in the CC domain and the final substitution was in the NB-ARC domain (Segretin *et al*., 2014). The mutation within the CC domain caused an increase in recognition of AVR3a^{EM}, although high levels of auto-activity were recorded in *N. benthamiana*. The single amino acid mutation located in the NB-ARC domain of R3a caused not only a gain of recognition of AVR3a^{EM}, but broadened specificity to include recognition of PcAVR3a4, an AVR3a homolog from *Phytophthora capsici* (Segretin *et al*., 2014). This change occurred in the predicted nucleotide-binding pocket, adjacent to one of the sensitizing mutations found in Rx by Harris *et al*. (2013), and produces a similar broad-recognition phenotype (Segretin *et al*., 2014). The authors suggest that the mutation in the NB-ARC domain gives rise to an over-sensitized mutant that could reveal cryptic activities of R3a (Segretin *et al*., 2014). A new R3a* mutant with a combination of the different amino acid changes in the LRR domain identified by Segretin *et al*. (2014) and Chapman and Stevens *et al*. (2014), whilst avoiding the mutations leading to auto-activity, could result in a stronger gain-of-recognition of AVR3a^{EM}.

It would be undesirable to deploy sensitised *R* gene mutants with broader resistance specificities in the field, as these could confer fitness costs to the plants. However, two naturally occurring amino acid mutations within the ARC2 subdomain of the *Pm3* resistance gene of wheat are known to confer broad-spectrum resistance to plants carrying these mutations (Stirnweis *et al*., 2014). This broad-spectrum resistance is associated with a fast HR and the authors infer that the ARC2 loop is an important

regulatory element of the PM3 protein (Stirnweis *et al*., 2014). With this in mind, future efforts at the directed evolution of *R3a* should, for example, target the CC-NB-ARC1/ARC2 domain of the immune receptor, in a bid to alter the resistance signalling response upon recognition of $AVR3a^{EM}$, in combination with the existing gain-of-$AVR3a^{EM}$-recognition mutations within the LRR domain. It is hoped that an intelligently evolved R3a gene, which provides recognition and resistance to *P. infestans* isolates harbouring either form of AVR3a, will deliver durable late blight resistance when deployed with other novel *R* genes.

4.5 CONCLUSIONS

The following conclusions can be drawn from the experiments detailed in this chapter:

a) R3a* shuffled variants Rd2-1, Rd3-1 and Rd4-1 are stably expressed *in planta*.

b) R3a* recognition of AVR3a^{KI} and AVR3a^{EM} is dependent on the molecular chaperones HSP90 and SGT1.

c) In a gain of mechanism, R3a* variants re-localise to late endosomes upon co-infiltration with AVR3a^{KI} or AVR3a^{EM}. A re-localisation to fast moving vesicles was also seen for AVR3a variants AVR3a^{KIL} and AVR3a^{EMG} as well as paralog PEX$^{147\text{-}3}$.

d) AVR3a^{KI} and AVR3a^{EM} re-localise to endosomes upon co-infiltration with R3a* variants but not, in the case of AVR3a^{EM}, with wild-type R3a.

e) R3a* shuffled variants maintain resistance towards an AVR3a^{KI}-expressing *P. infestans* isolate (7804.b) but have not gained resistance towards an AVR3a^{EM} homozygous isolate (88069).

CHAPTER 5

GENERAL DISCUSSION AND FUTURE WORK

5.1 GENERAL DISCUSSION

Although late blight disease contributed to the infamous Great Irish Potato Famine between the years 1845 to 1852, the pathogen remains an important threat to agriculture as it is estimated to cost the global potato industry in excess of £5 billion annually (Haverkort *et al*., 2009). Whilst developed nations are witnessing a decrease in their share of the potato market, the demand for the tuber in developing countries is driving global potato production (http://www.fao.org/potato-2008/en/world/). As fungicidal protectants remain prohibitively expensive for many farmers and some face being banned on environmental grounds, an alternative strategy for combatting late blight is needed.

Naturally occurring plant resistance genes have been bred into potato cultivars from wild crop relatives via traditional breeding methods and, more recently, by genetic modification (Jones *et al*., 2014). The heterozygous, auto-tetraploid nature of most potato varieties makes it very difficult to retain the commercially desired genetic background following a cross between a cultivar and a wild species (Haverkort *et al*., 2009; Jones *et al*., 2014). Traditional breeding methods are notoriously time-consuming, illustrated by the introgression of the more durable late blight resistance gene *Rpi-blb2* from a diploid wild species *Solanum bulbocastanum*. The development of the Bionica and Toluca potato cultivars took over 40 years from the first bridge cross between *S. acaule* and *S. bulbocastanum* in 1959 to the release of the cultivars in 2005

(Haverkort *et al*., 2009). Another problem associated with traditional breeding methods is the trend for introducing only one *R* gene at a time into cultivated potato, which has inevitably resulted in new races of *P. infestans* overcoming the newly deployed resistance in a relatively short period of time. Moreover, traditional crossing methods can limit the evolutionary potential of cultivars, as favourable combinations of alleles are broken up and there is great potential to introduce deleterious alleles of genes linked to the novel disease resistance (Jones *et al*., 2014). Conferring disease resistance to potato by genetic modification holds the advantage over more traditional methods, as multiple *R* genes can be stacked in high-yielding, favoured and well-established varieties to limit the chances of new races of *P. infestans* overcoming these new resistances.

One recent example of the genetic manipulation of potato is the introduction of the *Rpi-vnt1.1* gene from *Solanum venturii* (Foster *et al*., 2009) into the potato cultivar Desiree (Jones *et al*., 2014). During a three-year study, the stably-transformed GM plants were shown to have functional field resistance to the most predominant races of *P. infestans* (Jones *et al*., 2014). The commercial company J.R. Simplot is currently developing Innate^{TM} GM potatoes, which harbour *Rpi-vnt1.1*, in the USA (Clark *et al*., 2014; http://www.simplotplantsciences.com/index.php/generationtwo/overview). The genetic modification of crop plants opens up new possibilities for introducing novel disease resistance in potato. Not only can naturally occurring resistances be introgressed into existing cultivars, the wealth of information being produced from research into pathogen effectors can be utilised to intelligently design synthetic resistance genes (Farnham and Baulcombe, 2006; Harris *et al*., 2013; Stirnweis *et al*., 2014).

This thesis aimed to examine whether potato disease resistance gene R3a can be artificially engineered to provide recognition of and resistance to essential effectors from *P. infestans*. The R3a protein recognises the effector AVR3a^{KI} from *P. infestans* (Armstrong *et al*., 2005; Huang *et al*., 2005). However, the virulent form of this effector, AVR3a^{EM}, evades this recognition and promotes disease on plants harbouring R3a (Armstrong *et al*., 2005). AVR3a is an essential effector for *P. infestans* (Bos *et al*., 2010; Vetukuri *et al*., 2011) and is present in every modern *P. infestans* isolate sequenced so far (Armstrong *et al*., 2005, Cárdenas *et al*., 2011). There is limited sequence diversity of AVR3a in wild populations of *P. infestans*, with AVR3a^{KI} and AVR3a^{EM} forms being the most prevalent. Moreover, a weak R3a-dependent response to AVR3a^{EM} (Bos *et al*., 2006) suggests that this underlying, low level response could be enhanced to provide a stronger and faster resistance.

In addition to its avirulence activity, AVR3a^{KI} suppresses the cell death induced by INF1 elicitin (Bos *et al*., 2006). AVR3a^{EM} is able to weakly suppress INF1-mediated cell death, but not to the same high level as AVR3a^{KI} (Bos *et al*., 2006). An AVR3a isoform that is able to suppress cell death to the same level as AVR3a^{KI} cell death suppression, but evades recognition by R3a would be the most advantageous for *P. infestans*, but such a form has not yet been identified (Bos *et al*., 2009). A high-throughput structure-function analysis utilising saturated mutation of the C-terminal amino acid residues of AVR3a failed to identify such a form of AVR3a (Bos *et al*., 2009). The authors found that only a few mutations in AVR3a^{KI} result in loss of R3a activation without a negative effect on protein stability, resulting in a decrease in cell death suppression activity (Bos *et al*., 2009). Potentially, this study suggests that AVR3a would be unable to easily

evolve to evade a gain-of-recognition R3a* variant. Therefore, R3a is seemingly an excellent candidate for artificial evolution and this goal became the focus of this thesis.

Chapter 3 describes domain swapping experiments carried out between wild-type R3a and its paralog, R3a-Paralog1, and a non-functional homolog, DMG402027402, identified in the sequenced doubled monoploid *Solanum tuberosum* Group Phureja clone DM1-3 516 R44. The CC-NB domains of these two proteins share 96 % and 93 % amino acid sequence similarity with wild-type R3a, respectively. The native protein products of R3a-Paralog 1 and DMG402027402 were not able to recognise AVR3a. However, a chimeric protein consisting of the CC-NB domain of R3a-Paralog1 and the LRR domain of wild-type R3a, Pa1-R3a, was able to recognise AVR3a^{KI}. Moreover, chimeric Pa1-R3a conferred resistance to the AVR3a^{KI} homozygous isolate of *P. infestans* 7804.b, whilst a YFP-fused Pa1-R3a re-localised to late endosomes in the presence of AVR3a^{KI}, as does the wild-type R3a protein (Engelhardt *et al*., 2012). A chimeric protein made up of the CC-NB domain of the DM homolog DMG402027402 and the LRR of wild-type R3a showed only very weak recognition of AVR3a^{KI}, but no resistance or re-localisation in the presence of AVR3a^{KI}, possibly because this protein may be less stable *in planta*. This set of experiments demonstrated that only a 3 % change in amino acid sequence between the CC-NB domains of R3a-Paralog1 and the DM-homolog, relative to R3a, are needed to lose function. Reciprocal experiments to combine the CC-NB domain of wild-type R3a with the LRR domain of R3a-Paralog1 and the DMG402027402 homologs showed that both the R3a-DM and the R3a-Pa1 chimeric proteins were not able to recognise AVR3a. These results indicate that the LRR of R3a is involved in effector recognition to a certain extent, as function is

achieved only when the different domains of R proteins are attuned to recognition and signalling.

The results detailed in **Chapter 4** provide more evidence for the theory that co-evolution between the domains of R proteins is essential to achieve functional resistance against pathogens. R3a* shuffled variants harbouring mutations within the LRR domain of the protein showed a gain of recognition of the previously unrecognised AVR3a^{EM} effector form (Chapman and Stevens *et al*., 2014). Experiments characterising these R3a* variants revealed that, although the mutations in the LRR confer a gain of recognition and mechanism, these changes do not result in resistance to a virulent isolate of *P. infestans*. The mechanisms behind the recognition of AVR3a^{EM} by R3a* variants mimics those of wild-type R3a's recognition of AVR3a^{KI} in that SGT1 and HSP90 are required for the development of the HR. Furthermore, YFP-fusions of R3a* variants re-localise to PS1-labelled late endosomes upon co-infiltration with AVR3a^{KI} or AVR3a^{EM}, in the same manner that YFP-tagged wild-type R3a re-localises in the presence of AVR3a^{KI} only (Engelhardt *et al*., 2012). In ATTA experiments conducted in *N. benthamiana*, R3a* shuffled variants maintained resistance towards the AVR3a^{KI}-expressing *P. infestans* isolate 7804.b. However, R3a* variants did not confer resistance towards the AVR3a^{EM} homozygous isolate 88069. Stable transformation of potato plants with the R3a* variants recapitulated the finding that R3a* variants do not provide resistance to *P. infestans* isolates that are homozygous for AVR3a^{EM} (Chapman and Stevens *et al*., 2014).

NB-LRR proteins have been shown to localise to a variety of cellular compartments (Caplan *et al*., 2008). Over 80 % of cloned *Arabidopsis* NB-LRRs are predicted to have nuclear localisations, whilst a further 8 % are thought to be targeted to chloroplasts,

with only around 8 % predicted to be cytoplasmic (Meyers *et al*., 2003). The tomato I2 protein, a close relation of R3a, is thought to contain a nuclear localisation signal in its CC domain and the nuclear localisation of its cognate effector, Avr2, is required to trigger an I2-dependent cell death response (Simons *et al*., 1998; Ma *et al*., 2013). Although it is known that wild-type YFP-tagged R3a protein re-localises to late endosomes upon perception of AVR3a^{KI} (Engelhardt *et al*., 2012), the purpose of this cellular re-localisation remains elusive. Results detailed in **Chapter 4** (Chapman and Stevens *et al*., 2014) have added to this uncertainty, as R3a* variants also exhibit this re-localisation pattern in the presence of recognised effector forms, but do not confer resistance. There are a number of host proteins with which wild-type R3a is known to associate with, one of them being the host ubiquitin E3 ligase, CMPG1. Although CMPG1 is a virulence target of AVR3a, it is not required for the R3a-mediated HR (Bos *et al*., 2010; Gilroy *et al*., 2011b), demonstrating that it is not guarded by R3a. Two components of the exocyst, Sec3 and Sec5a, are known to interact with AVR3a in yeast-2-hybrid assays (Bos *et al*., 2010). Sec3 and Sec5a interact with each other and are involved in endocytosis and exocytosis (Sommer *et al*., 2005; Hála *et al*., 2008; Zhang *et al*., 2010) and thus may be associated with endosomal compartments in the endocytic pathway. Both Sec3 and Sec5a interact strongly with AVR3a^{KI} *in planta*, but only weakly with AVR3a^{EM} (Dr. Stefan Engelhardt, personal communication). A pyruvate-kinase-like protein, labelled KIPI30, has also been shown to form interactions with AVR3a. Moreover, silencing of KIPI30 *in planta* significantly increases the growth of *P. infestans*, potentially indicating that KIPI30 could be a virulence target of AVR3a. However, silencing of *KIPI30* failed to attenuate the R3a HR, indicating that it is not a guardee (Dr. Stefan Engelhardt, personal communication). Future work could involve investigating the interactions of the R3a* variants with Sec3 and Sec5a.

A recent study by Chapparo-Garcia *et al*. (2015) places AVR3a within a membrane complex that includes the vesicle trafficking protein GTPase dynamin-related protein 2 (DRP2). The authors cloned two DRP2 proteins from *Nicotiana tabacum* after AVR3a was found to co-immunoprecipitate with a homolog of the GTPase dynamin-related protein (DRP) in *N. benthamiana* (Chapparo-Garcia *et al*., 2015). The two DRP proteins have the classical five-domain structure of canonical dynamin proteins, whose functions in mammals include endocytosis and membrane remodelling (Chapparo-Garcia *et al*., 2015). The same study also found that AVR3a reduces the endosomal internalization of flg22-activated FLS2, but has no effect on the localisation of non-activated FLS2 (Chapparo-Garcia *et al*., 2015). AVR3a suppresses early defence responses mediated by the cell surface immune co-receptor BAK1/SERK3, as it reduced the production of ROS associated flg22 treatment in *N. benthamiana* (Chapparo-Garcia *et al*., 2015). This study provides more evidence that AVR3a is a multifunctional effector that can suppress PTI in different pathways (Chapparro-Garcia *et al*., 2015).

Although gain-of-AVR3a^{EM}-recognition R3a* variants have not been shown to confer resistance to AVR3a^{EM}-expressing isolates of *P. infestans* (Segretin *et al*., 2014; Chapman and Stevens *et al*., 2014), artificial evolution has proven to be a valid approach to creating novel disease resistance (Farnham and Baulcombe, 2006; Harris *et al*., 2013). Another well-characterised R protein-effector relationship, between potato R2 and AVR2 from *P. infestans*, presents an ideal case for artificial evolution to extend recognition specificity. *R2*, situated in the major late blight R2 resistance locus on potato chromosome IV, mediates recognition of AVR2, but another form, AVR2-like, is virulent on *R2*-carrying plants (Lokossou *et al*., 2009; Gilroy *et al*., 2011a). The two forms of *AVR2* differ in 13 amino acids, eight of which reside in the C-terminal effector

domain (Gilroy *et al*., 2011a). Avirulent *P. infestans* isolates are homozygous for *AVR2*, or heterozygous for *AVR2* and *AVR2-like*. Interestingly, some virulent isolates are known to carry both forms of AVR2, but AVR2 is transcriptionally silenced (Gilroy *et al*., 2011a). Three homologs of *R2*, *Rpi-blb3*, *R2-like* and *Rpi-abpt* (Park *et al*., 2005; Li *et al*., 1998), have similar recognition specificities to R2 (Gilroy *et al*., 2011a). A further seven R2 homologs, *Rpi-mcd1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-edn1.1*, *Rpi-hjt1.1*, *Rpihjt1.2*, and *Rpi-hjt1.3* (Vleeshouwers *et al*., 2011), are known to recognise AVR2 but not AVR2-like (Lokossou, 2010).

R2 is the biggest NB-LRR gene cluster in potato (Jupe *et al*., 2012; 2013) and harbours multiple functional resistance genes from diverse plants. There are a number of reasons that could explain this profusion of *R* genes: 1) these genes may be old and are therefore present in many plants; 2) *R2* homologs could have arisen independently, multiple times through duplication and diversification to recognise an important effector from a pathogen such as *P. infestans*; 3) different pathogens (or other effectors from *P. infestans*) could manipulate similar targets in the plants and these genes have evolved to maintain some kind of guarding function against the manipulation of the same pathway. Interestingly, *Rpi-mcq1*, a gene unrelated to *R2*, is able to recognise AVR2 through an unknown mechanism (Prof. Jonathan Jones, unpublished). This is further evidence that AVR2 is an important *P. infestans* effector or targets a critical host protein that the plant must protect.

The R2 protein indirectly detects AVR2 via an *in planta* association with the host kelch-repeat domain containing phosphatase, BSU-LIKE PROTEIN1 (BSL1), during infection by *P. infestans* (Saunders *et al*., 2012). Silencing of BSL1 attenuates R2-mediated recognition of AVR2 and resistance to AVR2-carrying *P. infestans* isolates (Saunders *et*

al., 2012). BSL1 was found to associate with R2, but only in the presence of AVR2, indicative of indirect recognition. AVR2-like also interacts with BSL1. However, it does not mediate the interaction of BSL1 with R2 (Saunders *et al*., 2012). Options for artificially evolving *R2* include introducing random mutations throughout the length of the resistance gene and then performing PCR shuffling on these mutants, before screening for gain-of-recognition of AVR2-like. This approach could potentially yield R2 variants with gain of recognition and attuned signalling to convert the novel recognition into resistance; a connection that is potentially still elusive in R3a* variants. However, a family shuffling approach could also be taken, where *R2* and its ten homologs are randomly fragmented before iterative rounds of PCR shuffling and screening are performed. The second approach may reduce the number of deleterious sequences produced as a result of the PCR shuffling process as natural selection would have pre-enriched these genes for functional diversity (Crameri *et al*., 1998). However, as all eleven genes mediate the recognition of AVR2, but not AVR2-like, there may be too little genetic diversity to produce shuffled sequences, which mediate a gain-of-recognition of the previously virulent effector. Rather than shuffle *R2*, an alternative approach could be to target the protein guardee of R2, BSL1. It is feasible that a mutant BSL1, which interacts strongly with AVR2 and AVR2-like as well as R2 and R2 variants, could confer durable resistance to AVR2-like homozygous isolates of *P. infestans*.

The artificial evolution of *R* genes is only one approach to creating race-specific or broad-spectrum disease resistance in plants. Other inducible layers of the plant innate immune system can also be targeted for manipulation to obtain more durable resistances. Mediators of the first layer of plant disease defence, the pattern

recognition receptors (PRRs), can be altered to provide increased levels of broad-spectrum disease resistance. It has been shown that a *Brassicaceae*-specific PRR, the receptor kinase EFR, which recognises the bacterial translation initiation-molecule elongation factor-Tu (EF-Tu) elicitor, can be transferred into a solanaceous host to confer resistance to bacterial diseases caused by *Ralstonia solanacearum* and *Xanthomonas sp*. (Lacombe *et al*., 2010). Recently, the receptor-like protein ELR from the wild potato species *S. microdontum*, which mediates extracellular recognition of the *Phytophthora* INF1 elicitin domain, has been demonstrated to weakly enhance resistance to *P. infestans* when transferred into cultivated potato (Du *et al*., 2015).

In addition to manipulating host receptors or host guardees, the identification of susceptibility (*S*) factors, host genes required for pathogen colonisation (Ekardt, 2002), provides a novel alternative for inducing resistance. However, much more research is needed to understand the nature of *S* genes, as many are thought to be involved in essential host processes and silencing of these genes may lead to lethal phenotypes (van Damme *et al*., 2009; Fawke *et al*., 2015). Nevertheless, conditional removal/silencing of *S* genes as well as the introduction of novel *S* alleles that maintain their endogenous function whilst not promoting pathogenicity may provide a suitable method of boosting disease resistance in crops (Fawke *et al*., 2015).

Finally, new genome editing tools such as Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based RNA-guided DNA endonucleases are promising to revolutionize the genetic engineering of organisms (reviewed by Gaj *et al*., 2013). One study demonstrated that by disrupting the rice bacterial blight susceptibility gene

Os11N3 (also called *OsSWEET14*) with designer TALENs, resistance to infection by pathogenic *Xanthomonas oryzae pv. oryzae* could be created (Li *et al*., 2012).

The greatest barrier to deploying durable disease resistance in crops is potentially the public's perception of genetically modified organisms. Genetic engineering is promising to enhance resistance to pests and diseases in agriculturally important crops, but acceptance and take-up of existing GM crops has been slow in European countries due to stringent EU regulations on the approval and labelling of new GM crops (Davison, 2010). With public opinion in the UK remaining mostly negative to GM foods (https://yougov.co.uk/news/2014/02/21/many-britain-remain-sceptical-gm-foods/), alternative methods that harness understanding of plant disease resistance are being developed.

Effector profiling of pathogen populations can inform decision making for *R*-gene deployment and fungicide application in current and subsequent potato growing seasons (Förch *et al*., 2010; Vleeshouwers *et al*., 2014). A scheme in the Netherlands informs farmers to spray their crops only when virulent alleles of effectors are detected in *P. infestans* populations. In addition to effector profiling, the spatially and temporally separated deployment of *R* genes may be able to avoid the rapid resistance-breaking ability of *P. infestans*. A programme in the Netherlands, named DuRPh (for Durable Resistance against *Phytophthora*), intends to deploy various combinations of stacked genes in different varieties at different sites at different times (Haverkort *et al*., 2009). These stacked *R* genes may be deployed as trans- or ideally as cis-genes (Haverkort *et al*., 2009; Park *et al*., 2009). Cis-genesis, the transfer of a naturally occurring gene to a recipient from a sexually compatible plant, may provide an alternative means of engineering disease resistance (Schouten *et al*., 2006; Park *et*

al., 2009; Haverkort *et al*., 2009). Although the methods used to create cis-genic plants are the same as those used for transgenic plants, cis-genes usually retain their own introns and remain under the control of their native promoters and terminators (Schouten *et al*., 2006). It has been argued that cis-genic plants should classified in the same way as conventionally bred plants and should be exempt from international regulations (Schouten *et al*., 2006).

5.2 FUTURE WORK

During the course of carrying out the experiments detailed in this thesis, ideas for other areas of work were developed. Listed below are some of the key questions that were left unanswered during my PhD, along with ideas for future work:

- As shown in **Chapter 3**, 33 amino acids were different in the CC-NB domain of the DM-homolog when compared to R3a and 32 amino acid changes when compared to the CC-NB domain of R3a-Paralog1. Selected mutation of these amino acids could give further clues to residues that are critical for signalling.
- The DM-R3a chimera described in **Chapter 3** consistently reduced the spread of *P. infestans* relative to the empty vector control in three independent ATTA experiments, although this effect was statistically non-significant. It would be interesting to include the native DM-DM protein and compare it to the DM-R3a chimera in an ATTA, to test whether the DM-R3a chimeric protein truly confers weak resistance to *P. infestans*. This study could also utilise selected amino acid substitutions from the experiment suggested above.

- The western-blot experiment described in **Chapter 3** needs to be repeated as only a single replicate demonstrated that the Pa1-R3a chimeric protein was stable *in planta*. Specific bands could not be detected in a further two replicates. It has proven very difficult to detect specific bands in western-blot experiments with wild-type R3a, chimeric R3a proteins and with the R3a* variants described in **Chapter 4**, in the presence of recognised effectors. Indeed, as it has been shown that upon recognition of an effector, the resistance proteins are re-localised to late endosomes, it is conceivable that they become bound in the insoluble pellet fraction during protein extraction. A potential experiment could be to assess the accumulation of vesicle bound R3a in the insoluble part by attempting to disrupt membranes further by boiling the insoluble fraction in SDS and then vortexing the sample.
- The finding that R3a* shuffled variants described in **Chapter 4** did not confer resistance to AVR3a^{EM} homozygous *P. infestans* isolates was very disappointing. However, it is clear that the NB-ARC domain could hold the key to achieving resistance and a stepwise approach to the artificial evolution of R3a needs to be taken. Mutations can be introduced to the NB-ARC domain in order to increase activation sensitivity of the resistance protein. These new mutations will be combined with the previously mutated LRR domain, ideally from the best-performing site-directed mutant Rd4-1, before screening for gain-of-recognition of AVR3a^{EM}. Amino acid substitution based on the comparison of the CC-NB domain of the DM-homolog to R3a and/or the R3a-Paralog1 CC-NB domain could be prioritised for this study.

- Selection for new R3a(NB-ARC)* variants with enhanced activation sensitivity will hinge upon the timing of the cell death response. Under the control of the native *R3a* promoter, R3a(NB-ARC)* variants can be screened by agro-infiltration in *N. benthamiana*. Co-infiltrations with AVR3a^{KI} and AVR3a^{EM} at low concentrations will show whether the new R3a(NB-ARC)* variants have a faster cell death response when compared to the speed of AVR3a^{EM} recognition afforded by Rd4-1. Importantly, all R3a(NB-ARC)* variants must be tested in the absence of the effector to identify auto-activators as mutations introduced in the NB-ARC domains of R proteins often lead to auto-activity.
- It would be interesting to test new R3a(NB-ARC)* variants for enhanced recognition specificity for other, unrelated effectors. Mutations within the NB-ARC domain of Rx have extended recognition to the distantly related PopMV virus (Harris *et al*., 2013), whilst Segretin *et al*. (2014) have shown that a mutation within the NB-ARC domain of an R3a* variant confers recognition of PcAVR3a4, an AVR3a family member from *P. capsici*.
- If new R3a(NB-ARC)* variants are shown to have faster, more robust cell death responses to AVR3a^{EM} in comparison to the original R3a* variants, ATTAs with AVR3a^{EM} homozygous isolates of *P. infestans* can be carried out in *N. benthamiana*, in the first instance. Stable transgenic potato plants carrying the R3a(NB-ARC)* genes could also be produced to test whether resistance is conferred to the natural host of *P. infestans*.
- If future R3a(NB-ARC)* variants are shown to confer resistance to AVR3a^{EM} homozygous strains of *P. infestans* in potato, this would be proof that artificial evolution can be utilized to extend the recognition and resistance to previously

virulent oomycete effectors. The argument for artificially evolving other resistance genes that recognise other essential *P. infestans* effectors is strong. As discussed earlier, the potato *R2* gene is an excellent candidate for intelligent evolution.

- Whilst the purpose of R3a re-localisation to late endosomes upon perception of AVR3a is unknown, it would be interesting to assay known host AVR3a-interacting partners with R3a* shuffled variants, as a number of these targets, such as Sec3 and Sec5a, are associated with endosomes and may thus act as guardees.
- It would be interesting to study if the re-localisation of R3a and R3a* to late endosomes is specific for R3a. For example I2, a close homolog of R3a has shown to be re-localised to the nucleolus upon perception of effector Avr2 from *Fusarium oxysporum*. R3b, which also resides on chromosome XI and is a member of the R3 cluster, shares 82 % nucleotide sequence identity and 73 % similarity at the amino acid level with R3a. However, R3a and R3b proteins have clearly distinct recognition specificities (Li *et al*., 2011). R3b recognises AVR3b from *P. infestans* (Li *et al*., 2011), whilst R3a detects AVR3a^{KI} (Armstrong *et al*., 2005). AVR3a is not recognized by R3b and AVR3b is not detected by R3a. Little homology exists between the LRR domains of R3a and R3b (67 % similarity at the amino acid level), compared with higher homology in the CC and NB domains (79 % and 75 %, respectively, at the amino acid level). It would be very interesting to examine and compare the cellular localisations of R3b in the presence of recognized effectors in experiments similar to those carried out by

Engelhardt *et al*. (2012). Similar localisation could give clues to the guardees of R3 family members.

- Other potential experiments include introducing mutations that are known to confer recognition of AVR3a^{EM} to the Pa1-R3a chimera and to an R3b-R3a chimera. There is the possibility that the domains in either one of these chimeras could be better attuned and are thus more likely confer resistance to AVR3a^{EM} homozygous *P. infestans* isolates.

CHAPTER 6

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